HEPATITIS B INFECTION:

IMMUNE MECHANISMS UNDERLYING ACUTE INFECTION

by

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Abstract

Following acute hepatitis B virus (HBV) infection, some individuals develop protective antibodies and recover. Others develop chronic hepatitis B (CHB), a leading cause of liver-related morbidity and mortality. CHB is characterized by intermittent hepatic inflammation and HBV-specific immune impairment. Current antivirals neither target HBV-specific immunity, nor eliminate the stable viral DNA within hepatocytes; thus, treatment is often lifelong. Improving our understanding of the immune mechanisms necessary to elicit recovery and overcome the immunotolerant hepatic microenvironment may facilitate development of a cure.

This thesis begins with a study of CHB prevalence in a Tibetan community living in Bylakuppe, India. Of 2,769 individuals tested, 247(8.9%) had CHB, and 613 (22.2%) had serological evidence of prior recovery from HBV infection, demonstrating that CHB remains highly endemic in some populations (Chapter 2).

In Chapters 3 and 4, immune mechanisms in acute HBV are examined to identify responses associated with recovery. To better understand the finding that the non-functional chemokine receptor CCR5 enhanced recovery from acute HBV in humans, *Ccr5*$^{+/+}$ and *Ccr5*$^{-/-}$ mice were intraveneously infected with an adenovirus containing the overlapping HBV-1.3 construct (AdHBV); intrahepatic immune dynamics were assayed by flow cytometry and ELISA. Following AdHBV infection, *Ccr5*$^{-/-}$ mice had enhanced infiltrating CD11b^{+} NK cells at Day 3 and pro-inflammatory CD11b^{+}Ly6c^{hi} monocytes at Day 14. Previous studies have demonstrated both NK cells and CD11b^{+}Ly6c^{hi} monocytes can improve HBV-specific T cell responses and facilitate recovery.

In Chapter 4, cytokines were assayed in individuals with incident HBV infection from the Multicenter AIDS Cohort Study (MACS). Of 110 HIV-uninfected individuals with incident HBV, 10 (9%) developed CHB. Elevated MIP1α, MIP1β, IP-10, IL-10, and IL-18 were observed during acute infection in HBsAg+ HIV-uninfected individuals with detectable antibodies to HBcAg (anti-HBc), but no differences were observed by HBV outcome. Interestingly, in HBsAg+ HIV-uninfected individuals tested prior to detection of anti-HBc, CCL17 was elevated in those that developed CHB. CCR4, a cognate receptor for CCL17, drives hepatic regulatory T cells recruitment, suggesting a mechanistic explanation for development of CHB. Together, these findings point to two potential targets for treatment of CHB (CCR4 and CCR5) that require future attention.

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CHAPTER 1:

Introduction to

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1.1. Hepatitis B Epidemiology and Clinical Features

Chronic infection with hepatitis B virus (HBV) is a leading cause of liver-related mortality worldwide, resulting from liver cirrhosis, hepatocellular carcinoma (HCC), and ultimately liver failure[1]. In 2015, an estimated 257 million people were chronically infected globally, leading to an estimated 887,000 HBV-attributable deaths[2]. Chronic hepatitis B (CHB) prevalence is unevenly distributed throughout the world, but is highly endemic (>8% prevalence) in the Asia-Pacific region and in sub-Saharan Africa (Figure 1.1).[3]

HBV is a blood-borne pathogen transmitted via exposure to infected blood or other body fluids. In endemic areas, HBV is commonly transmitted perinatally from mother to child during childbirth or from infected-individuals during early childhood[1,4]. In adults, transmission is often between unprotected sexual partners or via sharing of needles during injection drug use[5–8]. HBV is highly stable, persisting as infectious virus outside of the body for at least 7 days[9]. For this reason, it may be transmitted via sharing of razor blades, toothbrushes, or other objects that had been previously contaminated with infected blood[10,11].

Upon exposure, HBV has an incubation period of approximately 4 weeks before the appearance of detectable hepatitis B surface antigen (HBsAg) and HBV DNA[10]. Liver enzymes may become elevated around 1-3 months[12]. Symptomatic infection occurs in only 30-50% of adult-acquired infections; if they do appear, symptoms usually manifest around 3 months following exposure and may include jaundice, nausea, anorexia, fatigue, and fever[7,13]. In some cases, acute infection can lead to fulminant hepatitis and

acute liver failure. This is most common in older adults, where mortality rates from acute fulminant HBV infection are as high as 10-15% if untreated[11].

In approximately 90% of individuals exposed as infants and 5% of healthy adults, acute HBV infection progresses to the CHB[6,7,13–16]. Approximately 15-25% of untreated individuals with CHB will die prematurely from liver cirrhosis or cancer[17].

CHB has been categorized into four phases based on the status of the immune response and the presence HBsAg and hepatitis B e antigen (HBeAg) in the blood. These phases are called Immune Tolerant, Immune Active, HBeAg Negative Immune Active, and Inactive Chronic (Figure 1.3).[18] Those who develop protective antibodies against HBsAg (anti-HBs) are said to have recovered from the infection[11].

The Immune Tolerant phase is characterized serologically by a period of high HBV DNA (>20,000 IU/ml), normal levels of serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST), and circulating HBeAg[18]. The duration of this phase of HBV infection is longest in individuals infected in infancy or early childhood, where it can last until early adulthood[13]. It is characterized by the absence of a detectable inflammatory response, and is not associated with extensive liver damage[13,19].

An acute hepatic flare characterized by elevated ALT indicates progression from the Immune Tolerant phase to the Immune Active phase of CHB[13,18]. This phase of CHB is characterized serologically by high HBV DNA levels, detectable circulating HBeAg, and increased or fluctuating ALT/ AST. HBeAg+ disease is associated with higher HBV DNA levels, progressive liver damage and a greater likelihood of progression to cirrhosis and HCC[20].

Individuals who clear HBeAg from the circulation progress to either the HBeAg Negative Immune Active phase or the Inactive Chronic phase of CHB[18]. Individuals with HBeAg-Negative Immune Active CHB generally have less hepatic inflammation as compared to individuals with HBeAg-positive Immune Active CHB due to lower HBV DNA levels[20]. Still, individuals with HBeAg- Negative Immune Active CHB have some hepatic inflammation due to ongoing viral production and may result in progressive liver damage; individuals in the Inactive Chronic phase have limited viral replication and no hepatic inflammation, and thus experience little hepatic damage[21].

Recovery of HBV infection is defined serologically by undetectable HBV DNA and loss of circulating HBsAg, and is often accompanied by detectable antibodies against HBsAg[11]. Recovery occurs spontaneously in approximately 0.5-0.8% of individuals with CHB[22]. In some cases, occult HBV infection, defined by detectable serum HBV DNA in the absence of detectable HBsAg, may occur[23]. Reactivation of recovered or occult HBV has been documented in individuals with immune suppression following chemotherapy[24,25]. Thus, it is thought that HBV DNA is never fully eliminated from hepatocytes in recovered individuals; instead it persists as an episomal, covalently closed circular DNA (cccDNA, HBV life-cycle discussed in Section 1.3) that exhibits minimal HBV replication. Viral production may be detectable in liver biopsies but not in the serum.

It is estimated that HBV is the causative agent of 30% of cirrhosis and 53% of HCC cases globally[26]. A study of 3,653 Taiwanese individuals with CHB from the REVEAL-HBV cohort found male sex, increased age, elevated ALT, HBeAg positivity, and higher HBV DNA levels increased risk of progression to cirrhosis or HCC[27]. Further, progression to

HCC was also associated with a family history of HCC and a history of alcohol consumption[28–30].

Ten unique genotypes of HBV have been identified with between 4-8% sequence variation between the genotypes[31]. The geographical distribution and clinical manifestations of HBV vary by genotype. While genotypes A and D are most prevalent in the United States, B and C are the most common genotypes in China and most of Southeast Asia (Figure 1.3)[32]. Clinical outcomes tend to be worse in genotypes C and D, but genotypes A and B have been associated with greater rates of resistance to antivirals[32–34].

1.2 Hepatitis B Prevention and Therapy

Two recombinant DNA vaccines administered over a three-dose series have been approved for use, each targeting HBsAg with either aluminum hydroxide or aluminum hydroxyphosphate sulfate as an adjuvant[11]. A third, which can be administered in two doses over one month, was recently approved; this vaccine conjugates the recombinant viral antigen to a toll-like receptor 9 (TLR9) agonist[35]. Vaccination with any of the approved vaccines is >90% efficacious at inducing production of anti-HBs in those who receive the full dose schedule[11]. Vaccination strategies coupling administration of hepatitis B immunoglobulin (HBIG) with vaccination at birth have successfully reduced infection rates to 5-15% in children born in highly endemic areas[36]. Still, vaccination is not 100% effective, and HBsAg escape mutations have been documented, highlighting the need for effective treatment or a cure[37,38].

Several antiviral therapies, including pegylated-IFNα and drugs targeting the HBV reverse transcriptase (RT), have been approved for treatment of HBV infection. Pegylated-IFNα treatment rarely results in HBsAg seroconversion and is associated with adverse side effects, including flu-like symptoms and nausea[39,40]. Drugs targeting the HBV reverse trancriptase (RT) suppress viral replication and decrease hepatic inflammation, but do not eliminate the cccDNA from infected hepatocytes, so treatment is often life-long and may be out of reach for those in resource-limited areas. Further, drug resistance for many of the RT inhibitors has been documented, emphasizing the need for development of a cure[41].

Elimination cure can be defined as a complete eradication of hepatitis B cccDNA from hepatocytes, but this is rarely achieved in natural HBV infection, and thus may be a difficult endpoint to reach therapeutically[42,43]. Instead, a functional cure that mimics the immune control achieved by individuals who naturally recover from HBV infection is a more realistic goal. Still, a better understanding of the immune mechanisms underlying recovery, and strategies to elicit these mechanisms *in vivo*, will be essential to achieving a functional cure to hepatitis B infection.

This thesis will build upon the current epidemiological literature of HBV prevalence in a highly endemic, limited resource population (Chapter 2), highlighting the need for functional cures that mimic naturally occurring immunity. Additionally, this thesis will expand upon the current body of literature documenting immune function important to recovery from acute hepatitis B *in vivo* by characterizing cytokine profiles of acutely infected individuals associated with control (Chapter 4) and identifying the contribution

of CCR5, which has been linked to enhanced control of acute HBV in human genetic studies, to alterations of intrahepatic immune cells in a murine model of acute HBV (Chapter 3).

To orient the reader, the remainder of the introduction will be devoted to discussing the current knowledge of the virology and life-cycle of HBV (Section 1.3), the hepatic architecture and cell populations that create the immunotolerant liver environment (Section 1.4), *in vivo* and *in vitro* models for studying HBV (Section 1.5), immune cell function and the role of cytokines in hepatitis B infection (Sections 1.6 and 1.7, respectively), and the role of CCR5 in HBV and other hepatic conditions (Section 1.8). It will conclude with a brief discussion of the goals for the thesis (Section 1.9).

1.3 HBV Virus Structure and Life Cycle

Hepatitis B is an enveloped hepadnavirus, with a partially double stranded, relaxed circular DNA (rcDNA) genome[44]. The envelope consists of 3 surface proteins - the large, medium, and small HBsAg[45]. Interactions between envelope proteins and both heparin sulfate proteoglycans and the sodium taurocholate co-transporting peptide (NTCP) facilitate endocytic entry of HBV into hepatocytes[46–48] (Figure 1.4)[49]

Following entry, the HBV isocahedral nucleocapsid, which is formed from oligomers of hepatitis B virus core protein and encapsulates the HBV genome, is released into the cytoplasm where it traffics to a nuclear pore. Here, the nucleocapsid is degraded and the rcDNA is imported into the nucleus[50]. Inside the nucleus, the 3.2 kb rcDNA, consisting of a longer (-) strand and shorter (+) strand, is converted to cccDNA by host

DNA repair mechanisms[51]. The cccDNA serves as template for viral gene expression by host RNA polymerase, after which the viral mRNA is transported to the cytoplasm[52]. The 3.2 kb DNA virus has four partially overlapping open reading frames and four promoters (Pre-Core/Core, PreS1, PreS2/S, and X) which collectively encode the viral mRNAs, including the pre-genomic RNA (pgRNA) that serves as a template for viral replication[44]. The HBV genome encodes for 7 viral proteins- the large HBsAg, the medium and small HBsAg, HBcAg, HBeAg, HBV polymerase (HBV pol), and the HBV x protein (HBx)[53]. (Figure 1.5)[54]

To complete the viral life cycle, the 3' loop structure of pgRNA associates with the C-terminus of the core protein to facilitate encapsulation in the cytoplasm[55]. Inside the newly-formed capsid, the pgRNA is reverse transcribed by HBV pol to produce rcDNA[56]. From here, the viral nucelocapsid is either enveloped with HBsAg and secreted, or recycled back to the nucleus, where as many as 50 copies of cccDNA have been documented in a single hepatocyte[45,57,58].

One feature of HBV infection is that HBsAg can also oligomerize in the endoplasmic reticulum to produce empty subviral particles (SVP). SVP are often present in the serum of infected individuals at an excess of several logs over the infectious virus[59,60]. These SVP contribute to high levels of antigen (HBsAg) exposure in the periphery and subsequent immune dysfunction in individuals with CHB.

1.4 Hepatic Architecture

Located in the upper right quadrant of the abdominal cavity, the liver is the largest organ in the human body[61]. The liver is essential for bile production, elimination of toxins, lipid and cholesterol metabolism, protein synthesis, and carbohydrate homeostasis; more recently, the role of the liver as a unique immune organ has begun to be appreciated.

The liver is composed of parenchymal cells, namely hepatocytes and cholangiocytes (the epithelial cells of the bile duct), and non-parenchymal cells, including Kupffer cells (the tissue-resident macrophages of the liver), liver sinusoidal epithelial cells (LSECs), hepatic stellate cells (HSCs), and lymphocytes[62]. These cells are arranged into hepatic lobules composed of single-cell stacks of hepatocyte plates lining an extensive network of sinusoids and bile canaliculi. The lobules are organized around a central vein and surrounded by portal tracts containing branches of the hepatic artery, portal vein, bile ducts, and lymphatics[63] (Figure 1.6)[64]

The liver receives 20% of its blood supply via the hepatic artery and the remaining 80% from the portal vein, which carries blood rich in nutrients, toxins and antigens from the gastrointenstinal tract. The hepatic blood supply travels through the extensive network of sinusoids, where it is scavenged for nutrients and antigens, before draining into the hepatic vein and returning to the heart. Each sinusoid is surrounded by a discontinuous sheet of liver sinusoidal epithelial cells and Kupffer cells, which separates it from the perisinusoidal Space of Disse. Nutrients and antigens pass into the Space of Disse, where they are taken up via diffusion through epithelial fenestrae or by active transport

by LSEC[62]. Also within the Space of Disse reside the hepatic stellate cells, which are responsible for fat and vitamin A storage[65]. Bile is secreted from the basolateral membrane of hepatocytes, where it travels through an extensive network of bile canaliculi to the bile ducts of the portal triad. Surrounding the bile ducts are the large and small cholangiocytes[66].

Because the liver is constantly exposed to antigens from the gastrointestinal tract, it must maintain an immunotolerant environment that finely balances responsiveness to infection and avoidance of autoimmunity. Kupffer cells, or liver-resident macrophages, comprise approximately two-thirds of the non-parenchymal compartment, and account for 70% of the macrophage population in the body. Kupffer cells are responsible for phagocytosis of larger antigens (>200 μM) from the sinusoids. They are rich in TLRs and respond to antigenic stimulation, especially bacterial antigens, with robust production of IL-1β and TNF). LSECs, which line the sinusoids, are the most endocytic cell type in the body[5] and play a role in transcytosis of nutrients to hepatocytes[6] and of antigens for cross-presentation to T cells[67–69]. LSECs have high levels of TLR expression, and produce copious amounts of IL-6 in response to changes in lipopolysaccharide (LPS) concentrations[70]. IL-6 from LSECs induces hepatocyte activation to release acute phase proteins (APP) as well as facilitates a unique pathway of T cell activation[71,72].

Following activation by cytokines, hepatocytes are the primary source of proteins associated with the acute response to infection. Acute phase proteins include complement proteins, opsonin proteins, soluble factors for LPS signaling, and proteins associated with iron metabolism[73]. Of these, C-reactive protein (CRP) is important for

opsonization; it interacts with FcγRI and FcγRII on Kupffer cells and induces expression of IL-1β, IL-6, IL-18 and TNF[74,75]

Acute phase proteins stimulate increased expression of adhesion molecules on the surface of endothelial cells to enhance tethering of lymphocytes in the sinusoidal and peri-sinusoidal spaces via interactions with chemokine receptors, including CXCR3[76]. Specific lymphocyte populations within the liver and their role in hepatic immunity in the context of HBV infection will be further discussed in Section 1.6.

1.5. Model Systems for the Study of HBV Infection

Most studies of hepatitis B infection in humans are conducted amongst people who are chronically infected. From these studies, the HBV-specific immune impairment observed during CHB presents a chicken-and-egg dilemma: namely, do individuals with CHB develop immune dysfunction due to chronic antigenic exposure, or do individuals have early immune dysfunction that makes them increasingly more susceptible to development of CHB?

In other studies, acutely infected individuals were identified in the clinical setting following symptomatic presentation, biasing studies of acute HBV by exclusion of individuals with asymptomatic acute infections, the most common outcome of HBV exposure. To further confine human studies, sample collection is restricted to peripheral blood and rarely liver biopsies, which in turn limits the types of assays that can be performed and the understanding of intrahepatic immunity in HBV infection.

Much of our knowledge of the early immune dynamics of HBV infection is derived from the use of chimpanzees, which serve as natural hosts for HBV infection[77,78]. Tree shrews (*Tupaia belangeri*) are also susceptible to HBV infection, and studies in this model have recently shed light on the kinetics of the NTCP receptor for hepatocyte entry[79]. Variations of HBV, a member of the hepadnavirus family, are found in many organisms including duck HBV (DHBV), Beechey ground squirrels (GSHV), and woodchuck (WHV)[80–82]. Both WHV and GSHV have provided evidence that chronic hepadnavirus infection is associated with development of HCC[83,84], and the woodchuck model has been important to defining molecular pathways of HBV-associated carcinogenesis[85,86]. WHV and DHBV have been useful for *in vivo* and *in vitro* studies of viral dynamics (reviewed in Mason, 2015[87]) and efficacy testing of antiviral drugs[87–91]. While these animal models have been useful in identifying cellular subsets responsible for immune control of acute and chronic HBV infection [92,93], they are limited in their availability of immune reagents and inbred strains for the genetic manipulation of specific immune pathways to establish causality.

In order to take advantage of the wide array of reagents and inbred genetic backgrounds, several murine models of HBV were established. In the mid-1980s, transgenic mouse models containing either the full-length genome or genes encoding HBV viral proteins were developed[94–96]. While mice with a transgene encoding the full HBV genome produce large amounts of infectious HBV, they are immunotolerant to HBV antigens, likely resulting from early antigen exposure during thymic development[96]. Adoptive transfer of CD8+ T cells into transgenic mice helped identify the non-cytolytic

role of these cells in HBV infection[97–99], but this transgenic model was limited in that it did not mimic a true acute HBV infection.

To study the immune dynamics of acute infection, adenoviral delivery or hydrodynamic injection systems were employed to deliver the HBV genome into murine hepatocytes[100–102]. These mice produce infectious virions, present antigen to T cells, and are capable of producing antibody responses against HBsAg, mimicking human infection. Because the HBV that is produced in these models cannot subsequently infect neighboring hepatocytes, only a single round of infection is established, which means a narrow range in dose (~10-fold) differentiates acute and chronic infection. Further, cccDNA is not formed in these models. Chimeric murine models have been created by transplanting primary human hepatocytes (PHH) into immunodeficient uPA- or FAH deficient mice. These models have provided insight into the early innate and metabolic response to HBV in PHH, but are limited in their study of HBV specific adaptive immune responses[103–105].

In addition to animal models, several *in vitro* models have been developed to characterize the different stages of the HBV life-cycle, cccDNA dynamics, early innate responses, and drug efficacy. PHH would be the ideal candidate for *in vitro* infection assays, but they have poor stability *in vitro* and lose their cellular programming quickly following isolation, which renders them non-permissive to HBV infection. Further, there is high variability in PHH susceptibility to HBV infection by donor[106,107]. Micro-pattern co-culture (MPCC) systems, in which PHH are co-cultured with mouse fibroblasts, provide a platform for extended hepatocyte programming, which increases NTCP

expression and HBV-permissivity[108]. However, even in MPCC platforms, blocking of JAK-STAT signaling is necessary for HBV infection, a requirement that inherently skews immune responses of early HBV infection[109].

While traditional immortalized hepatoma lines such as HepG2 and Huh7 are not permissive to HBV infection, several hepatoma lines have been stably transfected with the HBV genome (Hep3B, HepG2.2.15, HepAD38, and HepDE19), and have provided systems to test drug efficacy and elimination of cccDNA[110,111]. More recently, the identification of NTCP as a receptor for HBV entry has led to the creation of stably-transfected HepG2-NTCP and Huh7-NTCP cell lines that overexpress NTCP and are permissive to HBV infection[46,47,112,113]. Use of these NTCP-overexpression lines permit *in vitro* study of the entire HBV life-cycle, as well as HBV-specific innate signaling within hepatocytes, but are limited in that they require a high MOI and have modest cccDNA production[47]

1. 6. Immune Function in HBV Infection

(As reference, characteristic B cell, T cell, and NK cell responses in acute and chronic HBV infection are summarized in Figure 1.7, which has been adapted in part from Maini and Pallet (2018)[114]).

1.6.1. B Cells

The primary role of B cells is to produce antibodies during infection, and the stages of HBV infection are characterized serologically by the presence or absence of antibodies

against HBcAg (anti-HBc), antibodies against HBeAg (anti-HBe), and anti-HBs. HBcAg is a robust activator of naïve B cells, which can activate B cells at extremely low levels without adjuvant in both a T cell-dependent and -independent fashion[115]. Due to its ability to crosslink surface receptors on naïve B cells[116–118], HBcAg induces large, albeit non-neutralizing, antibody responses during acute HBV infection in both individuals who recover and those that develop chronic, or persistent, infection. Anti-core IgM is detectable serologically at the onset of symptoms and can persist for six months following exposure in humans, and anti-core IgG antibodies are detectable years following acute infection and in individuals with CHB[11,119]. In individuals with a self-limited acute HBV infection, neutralizing antibodies against HBsAg appear in serological assessments around 3-4 months following exposure, or approximately 1 month following presentation of symptoms[10,11,119]. Antibodies against HBeAg may or may not appear, but often arise following hepatic flare and mark a transition in viral control from the Immune Active phase to either HBeAg-negative Immune Active CHB or Inactive CHB phase; individuals positive for anti-HBe tend to have lower HBV DNA and lower rates of progression to cirrhosis and hepatocellular carcinoma[20,21].

More recently, the immunoregulatory role of B cells in shaping T cells responses via production of cytokines, namely IL-10, has come into light. In 2012, Das et al. reported that individuals with CHB (10 HBeAg- and 5 HBeAg+) had greater numbers of IL-10-producing B cells following *in vitro* stimulation with CpG and PMA/Ionomycin compared to healthy controls[120]. Further, they found that HBcAg was able to induce IL-10 production in healthy donor PBMCs *in vitro*, and that the number of IL-10-producing B

cells, especially immature B cells, following HBcAg-stimulation *in vitro* increases in individuals with CHB during hepatic flares. These findings have been supported by findings from other groups, and support the notion that HBV skews B cells towards a more regulatory phenotype characterized by IL-10 production[121,122]

1.6.2. T cells

Cytotoxic T cells control viral infections by either direct killing of infected cells or by release of cytokines that indirectly kill infected cells or exert antiviral effects. Early studies in murine and chimpanzee models found that the non-cytotoxic functions of T cells, namely secretion of the pro-inflammatory Th1 cytokines IFNγ, IL-2, and TNF, play a crucial role in control of HBV infection[92,93,97–99,123].

Early studies in HBV transgenic mice found adoptive transfer of splenocytes from HBsAg-immunized donors or HBsAg-specific CD8+T cells led to a biphasic immune response. In the first phase, limited CTL-induced apoptosis was observed in only 2-10% of hepatocytes; instead CTL-derived IFNγ and TNF led to elimination of viral RNA, DNA, and HBcAg in the absence of robust liver injury[99]. The second phase was characterized by infiltration of a large number of non-antigen specific leukocytes into the liver and correlated with increased liver necrosis and ALT[124]. Studies of acute HBV in chimpanzees reported similar viral and immune kinetics, where control of infection relied on non-cytolytic control of HBV replication preceding a rises in liver damage as measured by serum levels of ALT[97,123].

Asabe et al. (2009) found that HBV viral inoculum determined T cell priming, the level of immunopathology observed, and final outcome in acute HBV infection[92]. In this study, high dose inoculum into chimpanzees (which had been the standard in previous studies) led to a characteristic pattern of HBV infection in which most hepatocytes were infected, and high levels of viral replication and expression were observed prior to elimination in a CD4+/ CD8+T cell-dependent manner. In chimpanzees receiving an intermediate dose, CD4+/ CD8+T cell responses controlled infection prior to detectable HBV DNA in the blood and without symptoms, while low viral inoculum primed the CD4+ T cell response towards a tolerant state that resulted in chronic infection. This study sheds light on the interplay between the size of the viral inoculum and T cell priming that are important to outcomes in acute HBV infection; these interactions likely plays a role in human infection, as inoculating dose has been shown to alter disease progression in humans[12].

Human studies have demonstrated that HBV-specific T cells are present in both acute infection and CHB, but that T cells from chronically infected individuals have a more exhausted phenotype, decreased functional capacity, and are more susceptible to deletion by receptor-mediated death, as summarized below.

HBV-specific CD4+and CD8+T cell responses directed against epitopes in the core antigen, HBeAg, polymerase and HBsAg have been detected by tetramer staining in individuals with acute, symptomatic HBV infection, many of which are detectable for years following control of infection [125–130]. T cells from individuals with acute HBV are able to produce cytokines and degranulate, which allows them to control infection[97,125].

HBV-specific T cells are also present by tetramer staining in individuals with CHB[130,131]. While HBV-specific responses following peptide stimulation are rarely observed immediately *ex vivo* in these individuals, this response can be improved following antiviral treatment[132]. HBV-specific T cells from individuals with CHB have higher expression of the inhibitory markers characteristic of an exhausted phenotype, including CTLA4, Tim3, and CD244, and greater expression of TRAIL and Bim, rendering them more susceptible to apoptosis[133–137]. The capacity of HBV-specific T cells from individuals with CHB to release perforin/granzyme and produce cytokines *in vitro* is improved by antibody blockade of PD1, CTLA4, and CD244[132,135,137]. A study comparing HBV-specific responses in peripheral versus intrahepatic CD8+T cells in CHB found that intrahepatic CD8+T cells produce IL-10 in response to HBV-specific peptide stimulation, suggesting that the liver microenvironment maintains CD8+T cells in an even greater anti-inflammatory state during CHB[138]. Consistent with this finding, deletion of regulatory T cells in a murine model of acute HBV infection lead to increased CD8+T cell functionality and decreased viral persistence[139].

1.6.3. NK Cells

Natural killer, or NK, cells are members of the innate arm of immunity and comprise a large niche within the steady-state liver microenvironment[140]. Activation of NK cells is dependent on a balanced interplay between engagement of activating and inhibiting surface receptors[141,142]. Once activated, NK cells have the capacity to induce cytolysis and produce cytokines[141].

Numerous animal studies have suggested a role for NK cells in non-cytopathic control of HBV via induction of IFNs. An early study of HBV in chimpanzees demonstrated IFNγ-mediated control of viral replication prior to the arrival of CD4+ and CD8+ T cells into the liver, implicating NK cells in the control of acute HBV infection[123]. Indeed, activation of intrahepatic NK T cells by α-GalCer in HBV transgenic mice led to rapid induction of type 1 and type 2 IFNs, recruitment of NK cells to the liver, and elimination of HBV replication even in the absence of T cells[143]. In a hydrodynamic injection model of acute HBV, PolyI:C treatment led to an IFN-dependent clearance of HBV[144], an effect that was significantly diminished by NK cell depletion[144,145]. Further, NK cells have been shown to modulate adaptive T cell responses in viral infections [146]. Zheng et al (2016) recently reported that NK cells enhance HBV-specific CD8+ T cell function in a murine model of HBV[147].

NK cells in patients who recover from acute HBV express a greater ratio of activation to inhibitory receptors and have increased cytotoxicity and IFNγ producing capacity[148]. Conversely, individuals with CHB have phenotypically impaired NK cell responses, characterized by increased expression of the inhibitory receptors Tim-3 and NKG2A and decreased capacity to produce IFNY and kill target cells[149–152]. Interestingly, flares in viral DNA during CHB are associated with decreases in the total number as well as the cytokine-producing function of peripheral NK cells[153,154]. Further, viral flares are associated with increases in serum IL-10 and decreases in the NK-cell activating cytokine IL-15[150,153]. Consistent with findings in individuals with CHB, murine studies have linked regulatory T cell-derived IL-10 to increased expression of the inhibitory receptor NKG2A

on NK cells, and have shown that blockade of NKG2A leads to HBV clearance in the HI model of HBV[149,155].

1.6.4. Myeloid Cells

Kupffer cells, the liver resident macrophage cell, are the primary antigen presenting cell of the liver, and maintain the immunotolerant state of the liver via secretion of IL-10 and TGFβ, which induces regulatory CD4+T cells[156]. Recently it was shown that HBcAg engages TLR2 signaling on Kupffer cells, which results in IL-10 production that can dampen HBV-specific CD8+T cell responses[157,158]. Indeed, Kupffer cells are often responsible for dampening immune responses by clearing apoptotic hepatocytes; in the absence of Kupffer cells, greater hepatic necrosis is observed, resulting in greater immune cell infiltrates, particularly polymorphonuclear neutrophils (PMNs) in transgenic mice following adoptive transfer of HBV-specific T cells[159].

Early studies in HBV transgenic mice demonstrated that there is a biphasic immune infiltration following adoptive transfer of splenocytes; during the second stage, non-antigen-specific monocyte-derived macrophages outnumber CD8+T cells by 100-fold and are responsible for increases in ALT associated with HBV infection[124]. Recently, pro-inflammatory monocytes within the liver in a murine model of acute HBV have been associated with local pockets of T cell priming and development of functional HBV-specific responses, suggesting an important role for these cells in initial innate responses as well as in the development of adaptive responses[160]. Unlike T cell responses, which are affected by constant exposure to antigen during chronic viral infections, monocytes

from individuals with CHB and healthy controls exhibit similar capacities to produce TNF and IL-6 following exposure to HBsAg[161].

1.7 Cytokines and Chemokines in HBV Infection

Cytokines play diverse roles in driving antiviral responses and inflammation in order to control infection, while also limiting immune-mediated damage. They enable signaling between cells to guide immune responses, recruit immune cells to sites of infection, and modulate gene expression within target cells, which may include antiviral effects. Several cytokines have demonstrated potent antiviral properties targeting different stages of the HBV lifecycle *in vitro* (Figure 1.8)[162,163], but these do not always translate to control of infection *in vivo*. *In vivo* studies in chimpanzees and murine models identified key roles for T cell-mediated control via TNF and IFNγ release, providing evidence that Th1-associated cytokines facilitate control of infection[93,123]. Since these early studies, others have demonstrated that a number of cytokines facilitate control of HBV infection both *in vitro* and *in vivo*. Given the myriad of cytokines that modulate innate and adaptive responses, it's likely that many of these associations are important, and cytokine production needs to be timed correctly during different stages of disease progression. The following section outlines the current understanding of the role of cytokines during HBV infection.

1.7.1. IL-1 Family Cytokines

The 11-member IL-1 cytokine family plays a key role in modulating inflammation and fibrosis. This family contains 7 pro-inflammatory cytokines (e.g. IL-1β, IL-18, and IL-33) and 4 anti-inflammatory cytokines or antagonist molecules (e.g. IL-37 and IL-1ra)[164]. IL-1β is a potent pro-inflammatory innate cytokine that acts locally and is rarely detected systemically. IL-1β is expressed in response to signaling via pattern recognition receptors (e.g. TLR4) as a pro-protein, which requires subsequent cleavage by inflammasome-activated caspases or other proteases (e.g. neutrophil elastase) to become mature IL-1β[165–168].

In vitro studies have demonstrated that IL1β can limit HBV infection by decreasing expression of NTCP, destabilizing cccDNA through the induction of the cytidine deaminase AID, and reducing viral mRNA through the induction of RNAses[169,170]. In humans, polymorphisms in the promoter region that result in increased expression of IL-1β (e.g. -511 C allele) have been associated with development of HCC in CHB[171], suggesting that although IL-1β may be helpful in limiting viral replication, prolonged IL-1β signaling can lead to enhanced immune-driven pathogenesis.

Like IL-1β, IL-18 is produced in a pro-protein form and requires cleavage by caspases to become mature IL-18[172]. While HBV outcomes may be associated with polymorphisms that control IL-18 expression, it is unclear whether HBV induces inflammasome activation and the release of mature IL-18[173–175]. *In vivo* studies in transgenic mice demonstrated that injection of IL-18 abolished rcDNA HBV within 24 hours[176]. In humans, higher IL-18 levels are associated with HBeAg seroconversion following

antiviral therapy in HIV-infected individuals with CHB[177]. This suggests that IL-18 may facilitate recovery in HBV infection.

1.7.2. IL-2 Family Cytokines

The IL-2 cytokine family consists of six cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) that share a common gamma chain and play an important role in T cell and NK cell activation and signaling[150,153,178,179].

Circulating IL-2 is increased, while IL-4 is decreased, during acute infection[180], suggesting a greater Th1 skew in acute HBV . Antiviral therapy restores circulating IL-2 in individuals with CHB[181], and likely facilitates in restoration of HBV-specific T cell function that is impaired in individuals with CHB. In CHB, decreased IL-5 and IL-7 are associated with HBeAg-positivity and high HBV DNA levels, respectively[182,183]. Finally, elevated IL-21 has been associated with HBeAg seroconversion and improved HBV-specific T cell function[184].

IL-4 drives Th2 responses alongside IL-13 and IL-5, which skews T cells away from the pro-inflammatory Th1 response thought to aid in control of HBV. IL-4 treatment of Hep3B cells has been shown to decrease HBV gene expression. Levels were not altered *in vivo* in humanized mice infected with HBV[185]. There is limited and conflicting evidence for an association between IL-4 and development of CHB[186–188], but one study suggests that circulating levels are affected by disease state in CHB[189].

IL-7 is crucial in differentiation of B cells, T cells, and NK cells, and in their maintenance and survival in the periphery[190]. Little is known about the role of IL-7 in acute HBV infection, but lower levels are associated with higher HBV DNA and HCC in CHB[183,191]. IL-15 is structurally similar to IL-2 and is important to activation and maintenance of T cells and NK cells[192]. It is typically presented in a trans-setting between cells, but cis-activation does have impaired function. Elevated IL-15 following peak HBV DNA is associated with recovery from acute infection[153]. In chronic infection, IL-15 is associated with HBeAg seroconversion following Telbivudine treatment[193]. In a mouse model, administration of IL-15 reduced plasma HBeAg and HBsAg in HBV-carrier mice[194].

1.7.3. Interferon Family Cytokines

Type 1 (e.g. IFNα, IFNβ, IFNλ) and Type 2 interferons (e.g. IFNγ) play a role in antiviral immunity[195]. Unlike other viral infections, HBV does not induce a robust type 1 IFN response[196]. It is unclear whether HBV actively suppresses IFN expression, or whether HBV acts as a stealth virus and evades the pattern recognition receptors responsible for IFN induction in viral infections. IFNα is used to treat HBV infection, but rates of HBsAg seroconversion are <10% after treatment [197].

In vitro studies have confirmed antiviral roles for type 1 and type 2 interferons in HBV infection. Both IFNα and IFNγ increase expression of APOBEC3A, which destabilizes cccDNA[198,199]. IFNα has been shown to limit transcription of HBV genes by deacetylating cccDNA and recruitment of transcriptional repressors[200–202]; it further destabilizes cytoplasmic pgRNA[203,204] . *In vivo* studies have demonstrated that T cell-derived IFNγ

reduces HBV mRNA in both transgenic mice and chimpanzees[123,205], and that combined with TNF, IFNγ is one of the main mechanisms of T cell-mediated noncytopathic control of HBV infection.

Increases in serum IFNα are observed in viral flares in chronic infection, and peak with ALT[206]. Serum IFNγ is increased in acute HBV infection and decreased in CHB as compared to healthy controls, but is associated with HBeAg seroconversion following IFN therapy in chronic infection[199,207].

1.7.4. TNF Superfamily

The TNF receptor family is composed of 19 ligands and 29 receptors. The cytokines TNF and lymphotoxin-alpha (LTα) both interact with TNF receptor 1, which has a cytosolic death domain, and TNF receptor 2[208].

CD8+T-Cell derived TNF was identified as an important mediator of non-cytotoxic control of HBV in early murine and chimpanzees studies[99,123,205]. Administration of TNF to transgenic mice led to decreased viral mRNA[99]. In vitro studies have demonstrated that TNF can inhibit HBV infection, downregulate NTCP expression, destabilize cccDNA by increasing expression of APOBEC3B, and reduce viral mRNA[169,170,199,209]. TNF is increased in PHH following HBV infection *in vitro*, and acute HBV is associated with increases in circulating TNF[199]. LTα increases APOBEC expression, but studies have been inconclusive on the role of this cytokine in inducing destabilization of cccDNA[198,210].

1.7.5. Other Cytokines

IL-6 is a pro-inflammatory cytokine that robustly induces the acute phase response in hepatocytes, but recent studies have identified additional roles for IL-6 in maintaining metabolic homeostasis via anti-inflammatory properties[211,212].

While *in vitro* studies have shown that IL-6 reduces transcription of HBV genes[170,213,214], *in vivo* studies found that IL-6 administration did not alter HBV mRNA in transgenic mice[99].

Serum IL-6 is increased in acute HBV in both humans and humanized mice and correlates with liver damage, but is lowered during resolution[185,215]. In CHB, spontaneous HBeAg seroconversion is associated with lower circulating IL-6[216], while HBsAg seroconversion following pegylated-IFNα therapy is associated with the soluble IL-6 receptor, which can act to bind to excess IL-6[216,217], suggesting decreased IL-6 is beneficial at later stages of HBV infection.

IL-12, a pro-inflammatory cytokine secreted by antigen presenting cells, acts upon T cells and NK cells to promote differentiation to a Th1 phenotype and subsequent release of pro-inflammatory cytokines, which are important to the non-cytopathic T cell control of HBV infection[98,99,218]. Administration of IL-12 alongside therapeutic HBsAg vaccination led to enhanced control of HBV infection in HBV carrier mice[219]. In CHB, higher IL-12 was associated with HBeAg seroconversion, and preceded induction of Th1 cytokines[207,220]. *In vitro* treatment with IL-12 decreased PD1 expression and rescued the functionality of HBV-specific CD8+ T cells from chronically infected individuals[221].

IL-10, the classic anti-inflammatory cytokine, was originally recognized for its capacity to inhibit T-cell activation, antigen presentation, and release of Th1 cytokines including IFNγ [222,223]. Consistent with its anti-inflammatory role, HBcAg-specific T cells from individuals with CHB secrete IL-10 following *in vitro* stimulation[224]. More recently, however, our understanding of IL-10 has become more nuanced, as immunostimulatory roles for this cytokine have been identified[222,223]. In CHB, HBeAg seroconversion is associated with increased circulating IL-10[220], and HBsAg seroconversion is associated with a single-nucleotide polymorphism in IL-10 that increases IL-10 production[225]. In acute HBV, serum IL-10 is increased and associated with higher circulating HBV DNA[153,180]. These findings suggest that IL-10 may be acting in a manner to encourage seroconversion and control, or it may simply be that IL-10 is enhanced to maintain homeostasis during inflammatory assault.

1.7.6. Chemokines

Chemokines are a family of cytokines that bind to transmembrane G-protein coupled chemokine receptors on the surface of immune cells in order to drive the chemotaxis of immune cells into sites of inflammation, and thus play an important role in ensuring proper immune responses[226].

Oo et al (2010) reported that the chemokine receptor CXCR3 is important for T cell homing to the liver, while other chemokines, such as CCR4 in the case of regulatory T cells, are responsible for localization of T cells once inside the liver[227]. Elevated levels of

IP-10, a CXCR3 ligand that is expressed in response to IFNγ, are associated with acute hepatitis B and HBeAg seroconversion following antiviral treatment of CHB[228–230].

The CCR5 ligands MIP1α and MIP1β have been shown to be important for HBV-specific T cell recruitment into the liver in HBV transgenic mice[231]. These CCR5 ligands have been associated with both acute HBV and with more advanced stages of HBV-related liver disease[229,232]. The role of CCR5 in HBV infection will be discussed in more detail below.

1.8 Chemokine Receptor CCR5 and Liver Disease

CCR5, a G-protein coupled chemokine receptor expressed on the surface of T cells, NK cells and macrophages, mediates immune cell trafficking into sites of inflammation by binding to its cognate ligands MIP1α (CCL3), MIP1β (CCL4), and RANTES (CCL5)[233]. CCR5 is most recognized for its role as a co-receptor for cellular entry by human immunodeficiency virus (HIV)[234]. Genetic studies found a 32 base-pair deletion in CCR5 (CCR5Δ32) that results in insertion of a premature stop codon following the fourth transmembrane domain and produces a non-functional CCR5 receptor. The CCR5Δ32 mutation, which occurs naturally in ~10% of Europeans, confers protection against HIV infection in those homozygous for the deletion, and resistance to infection or delayed onset of AIDS in individuals with one copy of the deletion[235].

A role for CCR5 in hepatitis B infection was identified by Thio et al (2007), who reported that the CCR5Δ32 mutation conferred protection against development of CHB following acute exposure[236]. Further, another mutation at -403A in the promoter of RANTES is

associated with increased circulating RANTES and enhanced control of HBV in the context of the CCR5Δ32 mutation, suggesting that excess RANTES in the absence of CCR5 signaling acts synergistically to alter immune responses to HBV[237]. CCR5 is not a cognate receptor for HBV infection, and thus it was hypothesized that CCR5 deficiency enhances recovery in acute HBV by altering the immune milieu in the liver during infection and subsequent development of immune response against HBV.

CCR5 binding has been shown to play a critical role in migration of memory CD8+T cell during viral infections and regulatory T cells in cancer. Less is known about the role of CCR5 in hepatic inflammation in different disease states, but the downstream effects of CCR deficiency are altered based on the specifics of the inflammatory insult.

Total numbers of NK cells, CD4+ T cells, CD8+ T cells, or NK T cells recruited into the liver following infection with LCMV clone 13 was not altered in *Ccr5*$^{-/-}$, *Cxcr3*$^{-/-}$ or *Ccr5*$^{-/-}$ */Cxcr3*$^{-/-}$ mice, but *Ccr5* $^{-/-}$ mice did have significantly enhanced steatosis, which was CD8+T cell-dependent, suggesting a differential skewing of the CD8+T cell response in *Ccr5*-deficient mice[238]. In a concanavilin A (ConA) model of T cell-mediated hepatitis, *Ccr5* knock-out (KO) mice exhibited more severe hepatitis due to increased RANTES production and hepatic recruitment of NK and T cells along the CCL5/CCR1 axis compared to *Ccr5* wild type (WT) mice[239,240]. By contrast, *Ccr5*- deficiency resulted in impaired NK cell trafficking into the liver and decreased intrahepatic IFNγ following *Toxoplasma gondii* infection[241].

1.9 Thesis Objectives

Recent years have brought a renewed interest for a cure for CHB. Due to cccDNA persistence within hepatocytes even after HBV recovery, a functional cure that mimics natural recovery by combining immunotherapy with traditional antivirals is likely more achievable than a cure that eliminates all cccDNA. While the introduction of cell lines permissive to HBV infection have accelerated *in vitro* drug discovery targeting the viral lifecycle, critical gaps in knowledge of immune responses important for recovery from hepatitis B, and thus important to informing immunotherapy strategies, still exist. Before addressing immune responses in hepatitis B, it is important to draw light on the epidemiology of this disease and the implications of vaccine and testing initiatives, as it is estimated only 9% of infected individuals are aware of their status. In chapter 2, a study of HBV prevalence in a Tibetan population living in Bylakuppe, India is presented. Three distinct populations are studied: the general community, a boarding school, and a monastery.

The CCR5Δ32 mutation is associated with enhanced recovery in acute HBV infection[236]. Maraviroc is an FDA-approved CCR5 inhibitor used in HIV care, and could provide useful in treatment of CHB, but more information is needed regarding the role of CCR5 in immune cell recruitment into the liver in hepatitis B infection. In Chapter 3, a murine model is employed to better understand the role of *Ccr*-deficency in hepatic immune cell trafficking and immune dynamics in acute HBV infection.

While CHB is associated with impaired HBV-specific adaptive responses, it is unclear whether these defects are present early in infection, as much of the knowledge of

immune responses in human hepatitis B infection has been conducted on samples from individuals with symptomatic acute HBV, or long after development of chronic infection. To address the paucity of knowledge on immune dynamics in early chronic, or persistent, HBV infection and asymptomatic acute infection in humans, Chapter 4 characterizes peripheral immune markers in a cohort of individuals with acute hepatitis B infection, some of whom recover from infection and others who develop persistent HBV.

Finally, Chapter 5 will offer a discussion of the findings, future directions, and perspectives on strategies for cure.

Figure 1.1: HBV worldwide prevalence

Image from Schweitzer et al. (2015)[3]

World map showing estimated HBV prevalence by country, gathered from a meta-analysis of studies conducted between January 1, 1965 to October 23, 2013.

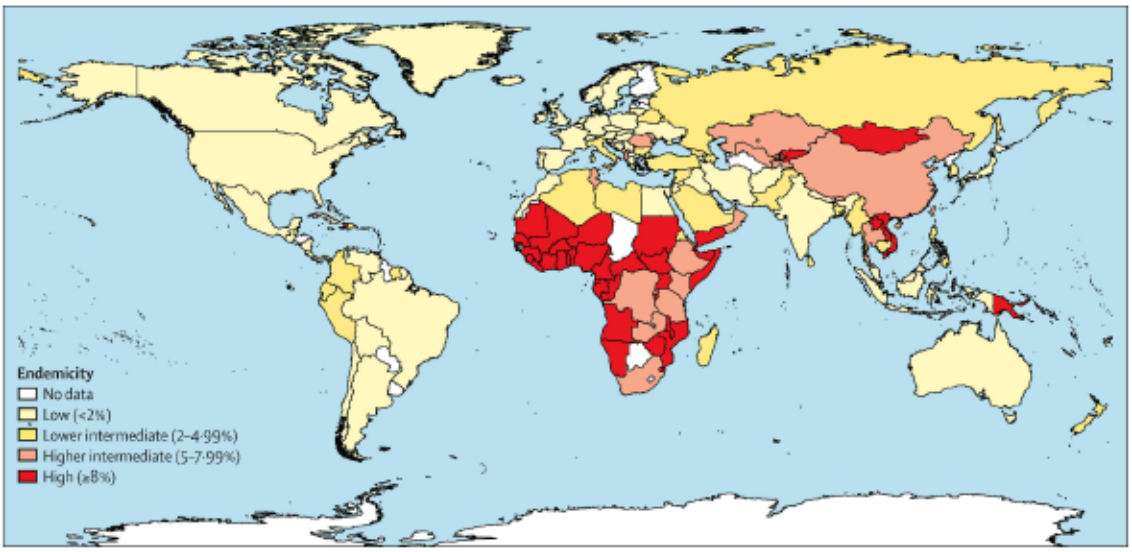


Figure 1.2: Phases of HBV infection

Adapted from Terrault et al. (2018)[18]

Phases of chronic hepatitis B (CHB) infection by serological status and treatment classifications. CHB can be divided into 4 phases: Immune Tolerant, Immune Active, HBeAg Negative Immune Active, and Inactive CHB.

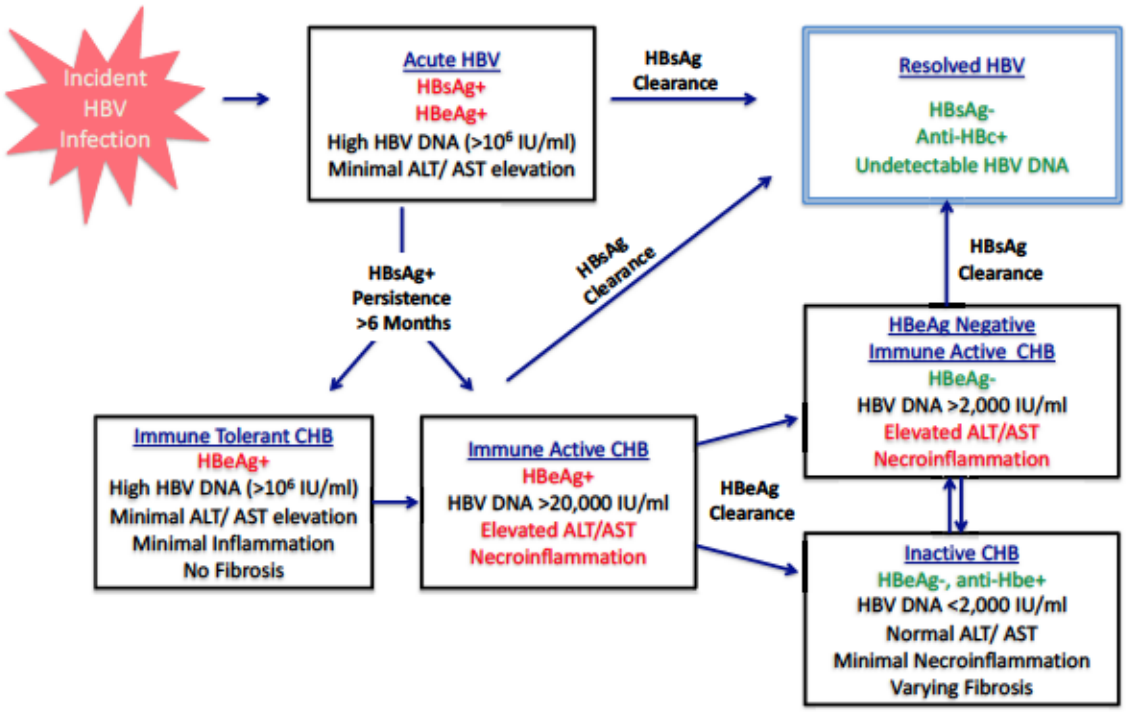


Figure 1.3: Global Distribution of HBV Genotypes

Image from Shi et al. (2013)[32]

World map showing global distribution of the different HBV genotypes.

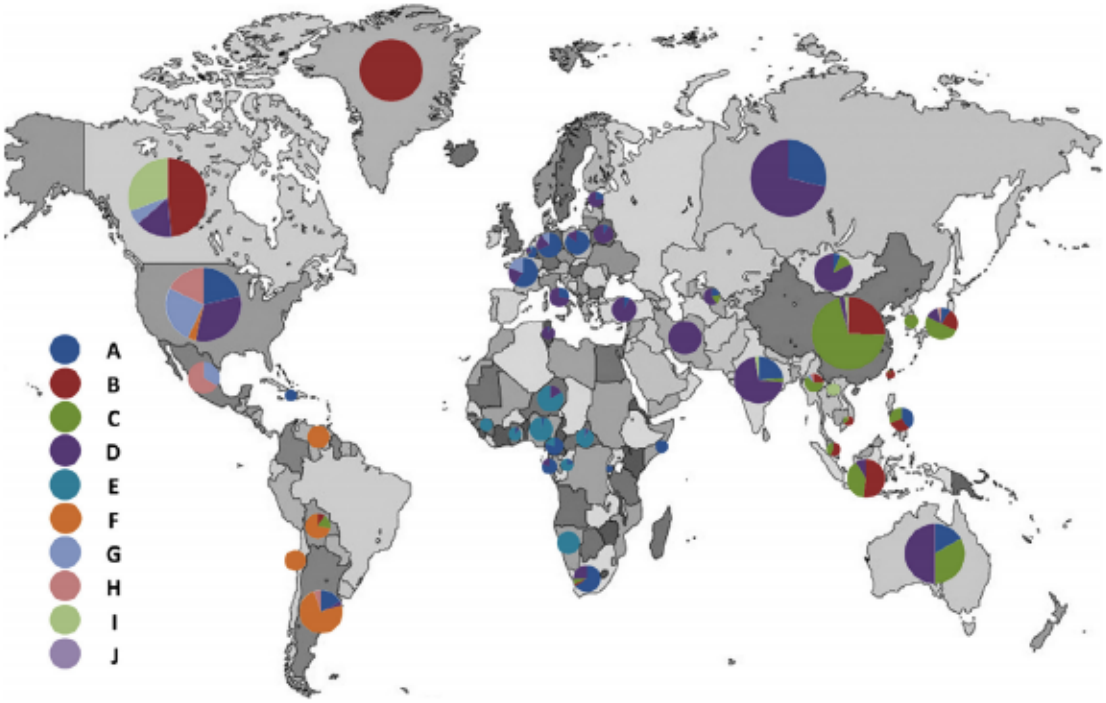


Figure 1.4: HBV life-cycle

Image adapted from Block et al. (2013)[49]

Hepatitis B envelope proteins bind to heparin sulfate proteoglycans and the sodium taurocholate receptor (NTCP) to enter hepatocytes. The capsid containing the rcDNA is released into the cytoplasm, and the rcDNA is transported to the nucleus. Within the nucleus, host repair machinery converts the rcDNA into a covalently closed chromosome, called the cccDNA. From the cccDNA, host machinery transcribes viral mRNA encoding viral proteins and the HBV pre-genomic RNA. In the cytoplasm, the virally encoded reverse transcriptase generates new viral DNA from pre-genomic RNA within newly formed capsid complexes. The capsid is either transported back to the nucleus to complete viral DNA replication, or enveloped and secreted as infectious particles.

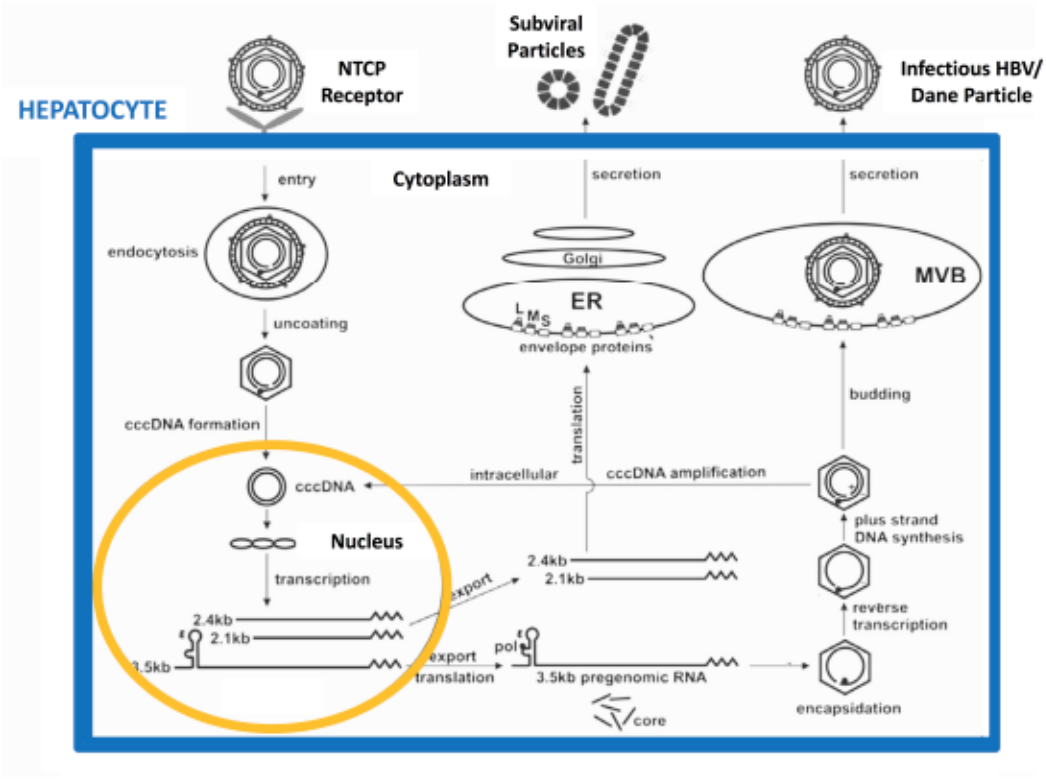


Figure 1.5: Hepatitis B Virus Genome

Image from Gish et al. (2015)[54]

The HBV genome is a circular double-stranded genome, with partially overlapping reading frames encoding for 7 viral proteins: large, medium, and small HBsAg, HBeAg, HBcAg, HBx, and polymerase.

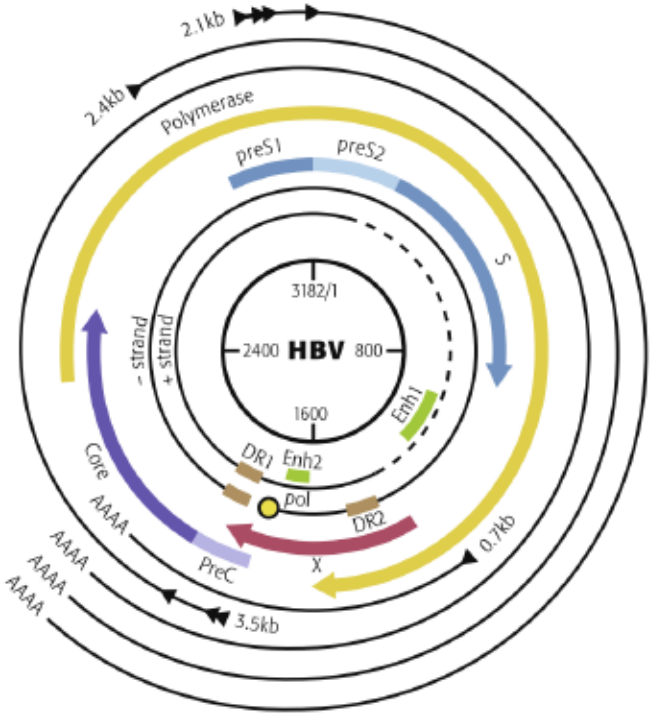


Figure 1.6: Hepatic Architecture

Image from Stenvall et al. (2014)[64]

Hepatic architecture showing organization of blood flow, lymphatics, and cells within the portal triads.

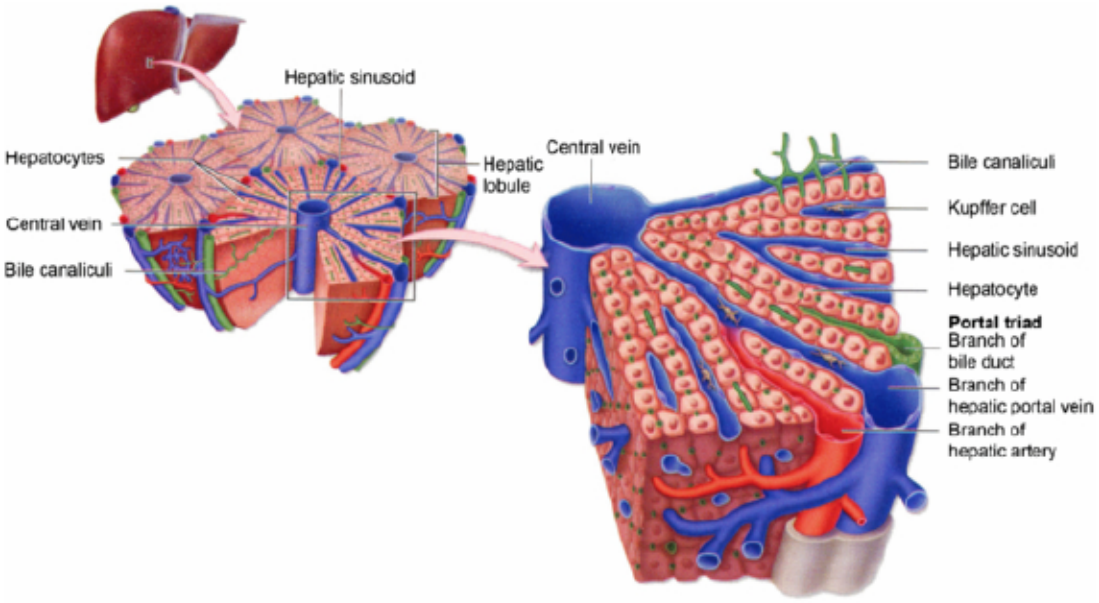


Figure 1.7: Immune cell characteristics in Acute and Chronic HBV

Adapted in part from Maini MK and Pallett LJ (2018)[114]

B cells, T cells, and NK cells all play important roles in recovery from acute HBV. B Cells are responsible for production of protective antibodies, CD4+ T cells prime CD8+ T Cells, CD8+ T cells exert non-cytopathic control of infection, and finally NK cells release cytokines and modulate T cell responses.

In chronic HBV, these cell types have impaired pro-inflammatory responses and increased regulatory or exhausted phenotypes, characterized by altered cytokine release and increased inhibitory receptors.

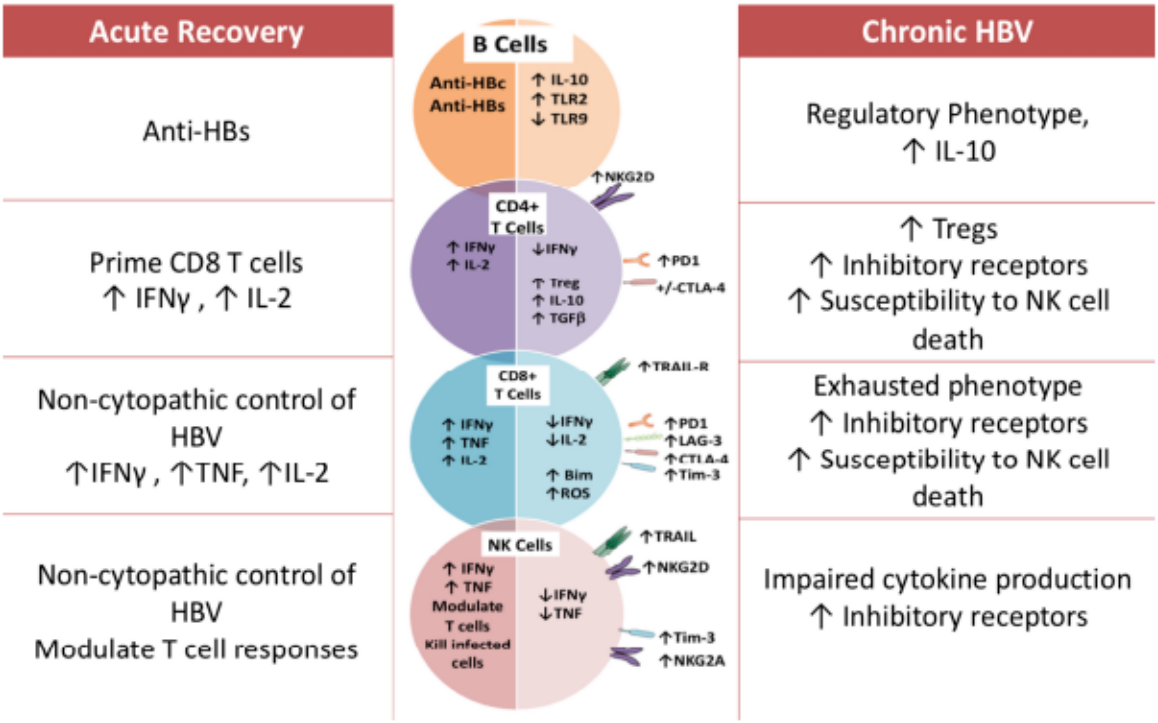
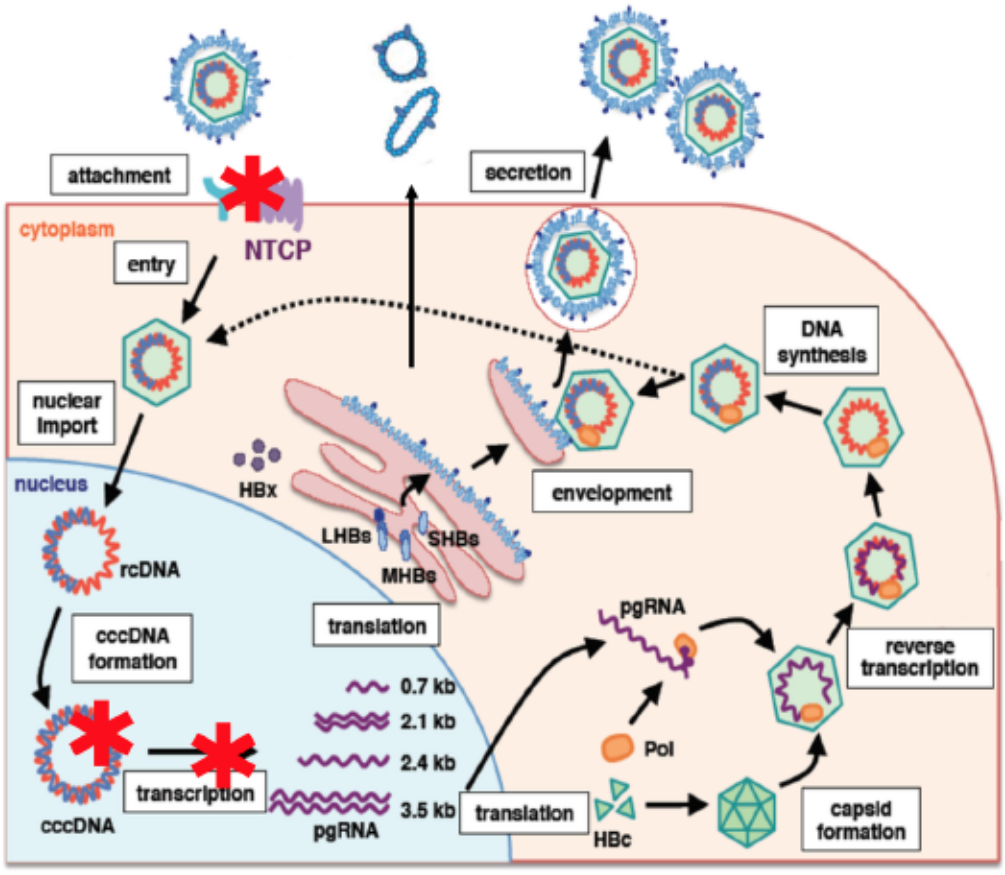


Figure 1.8: Cytokines and the HBV Life-cycle

Adapted from Xia Y and Protzer U (2017)[162]. Life cycle adapted from Watashi et al. (2014)[163]

Cytokines exert antiviral effects against HBV by downregulating expression of NTCP to prevent entry, inducing expression of deaminases to degrade cccDNA, and introducing epigenetic modifications that halt viral mRNA production.



	IFNα	IFNγ	TNF	IL-6	IL-1β
↓ NTCP Expression			+	+	+
cccDNA Degradation	+	+	+		+
↓ Viral mRNA	+	+	+	+	+

CHAPTER 2:

Hepatitis B prevalence and treatment needs among Tibetan refugees residing in India

This work has been previously published:
Stevens K, Palmo T, Wangchuk T, Solomon S, Dierberg K, Hoffmann CJ. Hepatitis B prevalence and treatment needs among Tibetan refugees residing in India. Journal of Medical Virology, 2016 Aug; 88(8): 1357-63.

2.1 Abstract

Untreated chronic hepatitis B can lead to liver failure and/or liver cancer. These complications can be avoided through prevention with vaccination or treatment of disease. To inform health policy for the Tibetan community in India, we conducted a cross-sectional study of hepatitis B prevalence and treatment needs over 3 months in 2013. Households were randomly selected for participation via a satellite map; one boarding school and one residential monastery were also included. Participants were asked questions and a whole blood sample was collected for HBsAg testing. Participants with a positive HBsAg result were tested for HBeAg, alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Participants with a negative HBsAg result were tested for anti-hepatitis B core antibodies (anti-HBc). We recruited 2,769 participants; of which 247 (8.9%) were positive for HBsAg. Participants more likely to have a positive HBsAg result were those born in Tibet (12.4%) and aged 30–59 years old. Of those with a positive HBsAg result, 60.7% were positive for HBeAg, of whom 7% fit into a likely treatment-needed category; the others fit into management categories requiring repeat ALT testing with or without liver fibrosis assessment. Among participants negative for HBsAg, 52.9% from household sampling had anti-HBc antibodies. We identified a high endemicity of chronic hepatitis B in a Tibetan community in India. Resource appropriate approaches are needed for managing chronic hepatitis B in settings such as this one.

2.2 Introduction

Hepatitis B Virus (HBV) infection is a serious global public health concern, with an estimated 240 million people chronically infected world-wide leading to 132,000 deaths annually[242]. Chronic HBV infection (CHB) is a leading cause of liver-related mortality resulting from liver failure, cirrhosis, and hepatocellular carcinoma [1]. Hepatitis B is uneven distributed globally and within sub-populations. East Asia and areas of Africa have a high endemicity of CHB, defined as a prevalence of 8% or higher. For example the prevalence is between 5% and 15% in the People's Republic of China, Taiwan, and Mongolia, and within immigrant communities from these regions, including Tibetans[243, 244]. CHB prevalence is generally lower (<5%) in Nepal, India, and Bangladesh[245,246]. In high endemicity regions of Asia, transmission is believed to mostly occur perinatally, from mother to child[1]. Transmission can also occur later in life through blood exposure, sexual intercourse, and use of needles for injection drugs or tattoos[247].

Acquisition of HBV can be prevented with vaccination and/or the use of hepatitis B immunoglobulin. Infant vaccination was introduced in India between 2002 and 2008[248]. Effective treatment is also available for individuals with CHB. The increasing availability of some medications, such as tenofovir, entecavir, and lamivudine, opens the possibility of providing affordable treatment for CHB in low and middle-income countries. In some situations treatment with interferon-alpha may also be a reasonable option. A clearer understanding of treatment needs, diagnostic limitations, and costs would allow health system planning for a rational approach to CHB prevention, diagnosis, and management.

The Tibetan diaspora is a population believed to have a high endemicity of CHB. Furthermore, large Tibetan communities exist in India (approximately 94,000 people), as well as smaller communities in Nepal, Bhutan, Australia, North America, and Europe, as a result of refugee flows from Tibet that started in 1959 and continues presently. We sought to determine the prevalence of CHB, identify subgroups with a higher CHB prevalence, and assess the treatment need among Tibetans living in India in order to provide data to assist in planning for prevention and treatment of CHB. We conducted a cross sectional study to estimate prevalence and management needs in a representative Tibetan community in the state of Karnataka in South India including random sampling from households and recruitment of participants from a boarding school and a monastery.

2.3 Materials and Methods

2.3.1 Participants and Study Design

We performed a cross-sectional study in Bylakuppe, Karnataka, India, which consists of 22 residential camps and a population of approximately 20,000 Tibetans. From July to October 2013 we recruited participants from three settings: households in the residential community, one boarding school (enrollment: 1,200), and one monastery (population approximately 1,300). Random household sampling was performed within the residential community using satellite images from Google Earth imported into ArcGIS with polygons drawn around each of the 22 residential camps forming the settlement. Random GPS coordinates were produced in each of the 22 polygons within

ArcGIS using python code (ESRI, Redlands, CA). We selected the three houses closest to the coordinates for sampling. All individuals residing in the household (defined as spending the prior night sleeping in the house) were invited to participate. All students and monks at the school and at the monastery, respectively, were invited to participate in the study.

The study protocol and consent procedures were approved by the Johns Hopkins University School of Medicine IRB and the Department of Health, Central Tibetan Administration. All participants completed written informed consent or ascent (if less than 18 years of age with a guardian signing consent).

2.3.2 Study Procedures

A brief questionnaire was administered to all participants in Tibetan by the study team. The questionnaire covered demographics, vaccination, and prior health conditions. A single venipuncture tube was collected from each participant.

2.3.3 Laboratory Methods

Rapid hepatitis B surface antigen (HBsAg) lateral flow testing (Alere Determine HBsAg, Alere, Waltham, MA, or SPAN Crystal HBsAg Device, SPAN Diagnostics, Surat, India) was performed within 5 hours of blood collection at the site of collection or at the local hospital laboratory on a serum sample prepared by centrifugation at 1,000g for 10 minutes in BD Vacutainer Serum Separation and Transport (SST) tubes (BD, Catalog #367983). Serum-separated samples were then transported to a research laboratory for

testing as follows: a sample with a positive rapid HBsAg test result had further testing for alanine transaminase (ALT), aspartate transaminase (AST), and HBeAg. Anti-HBc testing was completed for all community participants with a negative HBsAg test and a randomly selected (by random number generation) subset of monks and students with a negative HBsAg test. In addition, a subset of lateral flow HBsAg positive tests and 300 randomly selected HBsAg negative tests were confirmed by laboratory-based HBsAg ELISA to assess the accuracy of the rapid tests.

2.3.4 Definitions

We used a single HBsAg positive test as a surrogate for CHB. We acknowledge that the clinical definition of CHB is two positive HBsAg tests at least 6 months apart but selected a single test because most individuals are expected to have been infected early in life and it is unlikely that many or any of the positive HBsAg tests reflected acute hepatitis B infection and it is consistent with prior recent studies of CHB epidemiology[249–254]. Prior exposure was defined as HBsAg negative and anti-HBc positive. Individuals were classified as never infected if they were HBsAg negative and anti-HBc negative. Prior HBV vaccination was based on self-report (confirmed with a vaccination card when available).

ALT and AST normal range was based on the laboratory test reference range, which defined 40 IU/ml as the upper limit of normal. We classified participants with CHB into management categories based on recommendations from the American Association for the Study of Liver Diseases[14] (Table 2.1). In the absence of HBV DNA data we followed

the "HBeAg positive pathway" classifying into three categories based on ALT. We selected this classification system rather than more current classifications because we lacked HBV DNA data, a primary consideration in contemporary guidelines[255,256, 14]. We selected to use an approach not requiring HBV DNA results because of the cost and limited availability of HBV DNA testing for routine use in some low and middle-income settings, including India[257].

2.3.5 Statistical Analysis

Chi-square tests were employed to assess associations between of HBV status and participant characteristics. Stepwise multiple logistic regression was performed to determine the odds ratio for infection given participant characteristics. We performed separate logistic regression analysis for the community and the school and monastery because we used random sampling with the community; whereas, we invited all monastery and school residents to participate and did not attempt representative sampling. All analyses were two-sided, with a $p<0.05$. Models were assessed for goodness of fit using the Hosmer-Lemeshow test. We calculated sensitivity and specificity with 95% confidence interval intervals for the rapid SPAN Crystal HBsAg Device against a laboratory ELISA using the efficient score method corrected for continuity[258]. We did not assess test performance for the Alere Determine rapid test, as validation studies were publically available. Statistical analysis was performed using STATA 13 (StataCorp. College Station, TX).

2.4 Results

2.4.1 Demographics

We recruited 2,769 participants, 945 (34.1%) were from 299 randomly selected households (size ranged from 1 to 11 family members), 1,153 (41.3%) were from the boarding school, and 671 (24.6%) were from the monastery. Three households declined participation. Monks and students were passively recruited and thus non-participation was not determined for these groups. Overall, the median age was 18 years with a range of 3 months to 94 years; 61% were men (due to recruitment in the monastery; Table II). The majority of participants were either born in India (1,466, 52.9%) or Tibet (1,122, 40.5%). Age differed by country of birth: the median age among those born in India was 16 years (interquartile range [IQR] 11–24), in Tibet was 28 years (IQR: 18–56), and in Nepal was 15 years (IQR: 12–18).

2.4.2 Chronic Hepatitis B

A total of 247 participants (8.9%; 95%CI: 7.9, 9.9) were positive for HBsAg. Focusing just on the household sampling, 11.9% (95%CI: 9.9, 14.1) were positive for HBsAg. HBeAg testing and ALT and AST were performed on 244 of 247 HBsAg positive individuals. Three participants were excluded from this testing due to insufficient sample volume. One hundred and forty eight (60.7%) HBsAg positive participants were HBeAg positive. Among HBeAg positive participants, 26% (43/146) had an ALT above the upper limit of normal compared to 19% (18/97) of HBeAg negative participants (chi- square, P= 0.06). Only 7% (17/244) of participants had an ALT >2 times the upper limit of normal.

The median age of HBsAg positive participants was 30 years (IQR: 18–44), compared to 18 years for uninfected individuals (IQR: 13–34). Infection rates were slightly, but non-significantly higher in males than females, and greatest among individuals between the ages of 30–59 (Table 2.3). HBsAg positivity was higher in those born in Tibet than those born in either India or Nepal (12.4% vs. 6.8% vs. 2.4%, $P < 0.001$).

In multivariable logistic regression analysis restricted to the household, individuals reporting a family history of hepatitis B diagnosis were 3.4 times (95%CI: 2.0–5.6) more likely to have CHB (Table IV). Age and self-reported HBV vaccination were also associated with CHB status with individuals aged 30–59 most likely to have CHB with an odds ratio of 10.4 (95%CI: 2.4–45) when compared to those <15 years old. The odds ratio for chronic hepatitis B among those with self-reported vaccination was 0.083 (95%CI: 0.05–0.14) compared to those that did not report vaccination. In further assessment of the household data, CHB prevalence was higher among individuals who had a household member also positive for CHB was 19.3% compared to 9.4% if no other household members tested positive ($P<0.001$).

2.4.3 Prior HBV Exposure

Prior HBV exposure with subsequent control (anti-HBc positive and HBsAg negative) was identified among 613 (22%; 95%CI: 21, 24) participants. Among the household population 475/945 had evidence of prior exposure (50.2%); 235 (71%) of those born in Tibet and 227 (39%) among those born in India had evidence of prior HBV exposure. Among the household sample, when combining participants positive for either HBsAg or

anti-HBc antibody, 62% (95%CI 59, 65) either had current CHB or controlled infection (Figure 2.1).

2.4.4 Hepatitis B Treatment Needs

Of the participants we identified with CHB and an age greater than 15 years, 16 (7%) had an ALT greater than two-times the reference range, suggesting an indication for treatment[259]. Another 37 (17%) had an ALT elevation between one and two time the reference range, placing them in the category of re-test ALT in 3 months and assess for liver fibrosis to guide treatment. The majority, 163 (76%), of participants had normal range ALT placing them in the category of monitoring ALT again in 6 months and only evaluating further and considering treatment if the ALT were to rise above the upper limit of normal.

For the SPAN rapid HBsAg test, we calculated a sensitivity of 99% (95%CI: 97, 100; 197 positive rapid tests of 198 positive by ELISA) and specificity of 100% (95%CI: 97, 100; 176 negative rapid tests of 176 negative by ELISA).

2.5 Discussion

Overall prevalence of hepatitis B infection was 8.9%, with 11.9% observed in the household sampling. Prevalence was higher in individuals over the age of 15 years, with the highest rate of infection seen in participants between the ages of 30 and 59.

Our findings are consistent with a convenience sample study of Tibetan exiles living in Nepal which found 16% with CHB and 45% with positive testing for anti-HBc.[243] Another

study completed among Tibetans in villages in China that reported a 21% prevalence of CHB[244]. This is in contrast to the CHB prevalence of 2–3% among the Indian population in India[260]. We identified important associations of a higher prevalence of HBV exposure and CHB with birth in Tibet. The potential for a higher CHB prevalence among people living in Tibet was previously suggested by convenience sample testing in Tibet in which the 26% of participants were HBsAg positive[261]. Part of the association we observed can be attributed to the older age of participants born in Tibet.

We believe that our findings reinforce the importance of birth HBV vaccination (which is current policy in India) as well the importance of increasing access to hepatitis B immune globulin for prevention of mother- to- child transmission. Higher HBV prevalence in the age group between 15 and 59 may be an indication of ongoing HBV transmission among the population born before the start of routine infant HBV vaccination in this population. If so, adolescent and young adult catch-up vaccination may be prudent. However, in a cross sectional study such as ours, we are unable to distinguish between ongoing exposures versus a cohort effect to explain the increasing HBV prevalence with age. Family history was also strongly associated with current and previous hepatitis B infection. Those with another member of their household who tested positive for HBsAg were more likely to also be currently infected with CHB, presumably either a result of peripartum or household transmission.

Tibetans in this population generally give birth in hospitals where vaccination and hepatitis B immune globulin are available. However, transmission is not always prevented—as observed in our study with at least one probable case of mother to child

transmission in a 3-month-old infant positive for HBsAg. Consideration for maternal treatment with antiviral medication may be reasonable to prevent these infections. Previous studies have found that 8 weeks of lamivudine treatment in late pregnancy significantly lowers maternal HBV DNA and reduces the risk of HBV transmission[262]. Using the AASLD recommendations[14], approximately 1% of the total adult Tibetan population in our survey fits into the category of needing treatment for CHB (after confirming sustained ALT elevation). For another 2% of the adult population, follow-up ALT and liver histology testing is indicated to determine treatment need. Finally, to adhere to guidelines, 8.8% of the adult population should have routine 6-monthly follow-up to monitor for inflammation, fibrosis, and hepatocellular carcinoma. These findings highlight the range of management needs of individuals diagnosed with CHB, the complexity of applying current management guidelines in settings where medical costs of HBV DNA testing are prohibitive (guidelines available from the Asian Pacific consensus on CHB treatment, European Association for the Study of Liver Disease, and others have similar algorithms and complexity[255,256,259], and the large proportion of the population who need long-term medical follow-up based on guidelines. Delivering the recommended level of laboratory testing and antiviral treatment to 10% of the adult population of Tibetans in India (and likely elsewhere in the diaspora) with CHB will require allocation of considerable resources. If we extend these percentages to the approximately 94,000 Tibetans living in exile in India with an estimated one-half over the age of 15, approximately 8000 would need repeated ALT and/or liver fibrosis

assessment, and 360 adults are likely to currently meet recommendations for CHB treatment based on HBsAg and HBeAg positivity and an elevated ALT.

Our study has the strength of representative sampling for the household survey based on random selection and very high participation. We did not use representative sampling for the school and monastery. As a result, the prevalence figures could underestimate the true prevalence because individuals with a known CHB diagnosis may not have sought testing. This is less likely to have occurred at the school where testing had not been previously provided. We believe that the results also reflect the epidemiology among other Tibetan communities in India, in the greater diaspora, and possibly also in greater Tibet. Limitations are those of cross-sectional studies, including inability to contribute further understanding to timing of HBV transmission within this community. In addition, we lacked liver biopsy and HBV DNA results, both of which could be valuable for treatment staging.

We have identified the high prevalence of CHB and substantial HBV treatment need within a Tibetan community in India. As the only study to systematically determine prevalence and treatment needs in this population to date, we believe that we have identified an important public health need in this population. However, further work is needed to improve low-cost tools for staging and monitoring treatment to provide access to the HBV care services for this and similar populations. Improving access to medications for the treatment of CHB to indigenous and displaced communities and those in settings with constrained resources may be especially important for reducing HBV-related morbidity, mortality and HBV transmission globally.

Figure 2.1. Hepatitis B Status by Sampling Population

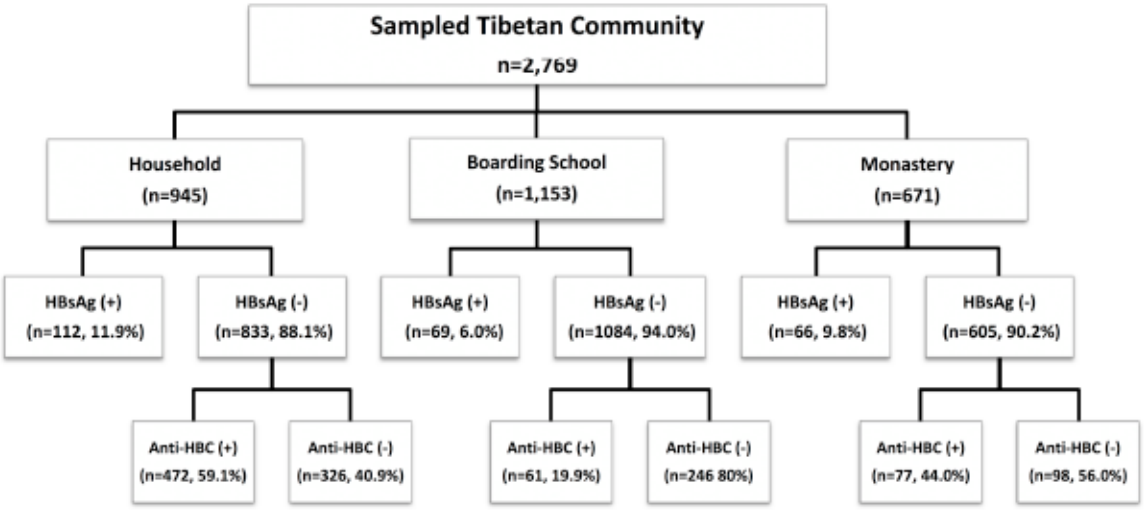


Table 2.1 Chronic Hepatitis B Treatment Recommendations (Summarized From the AASLD)[20]

HBeAg Status	ALT	Additional Testing	Treatment Recommendations
HBeAg (+)	ALT > 2x ULN	Repeat ALT in 1-3 months	Treat if ALT remains elevated or evidence of liver disease
HBeAg (+)	ALT 1-2X ULN	Repeat ALT testing in 3 months; if >40 years old & ALT remains 1–2 ULN, consider testing for liver fibrosis	Depends on repeated ALT results
HBeAg (+)	Normal ALT	Repeat ALT in 3–6 months, repeat HBeAg in 6–12 months	Depends on repeated ALT results
HBeAg (-)		Obtain HBV DNA levels	Depends on HBV DNA level

Table 2.2 Participant Demographics by Sampling Location

	Household, N (%)	Boarding School, N (%)	Monastery, N (%)	Total, N (%)
N=	**945**	**1,153**	**671**	**2769**
Sex, Male	455 (48%)	561 (49%)	671 (100%)	1680 (61%)
Age (years)	**42 (IQR=23-61)**	**16 (IQR-12-18)**	**22 (IQR=15-33)**	**18 (IQR=14-36)**
<15	162 (17%)	464(40%)	165 (24.6%)	791 (28.6%)
15-29	139 (15%)	689 (60%)	280 (41.7%)	1108 (40.0%)
30-59	395 (42%)	0 (0%)	214 (32%)	609 (22%)
>60	249 (26%)	0 (0%)	12(1.8%)	261(9.4%)
Birth Location				
India	581 (62%)	641 (55%)	244 (37%)	1466 (53%)
Tibet	331 (35%)	471 (41%)	320 (48%)	1122 (41%)
Nepal	2 (0.2%)	24 (2.1%)	97 (15%)	123 (4.4%)
Unknown	31 (3.2%)	17 (1.5%)	10 (1.5%)	65 (2.3%)

Table 2.3. Characteristics by Hepatitis B Surface Antigen Status

	HBsAg Negative (n=2522) N (%)	HBsAg Positive (n=247) N (%)	P
Age (years), median (IQR)	18 (13-34)	30 (18-34)	0.224
Age Category			**<0.0005**
<15	770 (97%)	21 (2.6%)	
15-29	1010 (91)	98 (8.8%)	
30-59	512 (82)	109 (18%)	
>60	230 (92%)	19 (7.6%)	
Gender			0.1
Male	1518 (90%)	162 (9.6%)	
Female	987 (92%)	84 (7.8%)	
Sampling Location			**<0.001**
Community	833 (88%)	112 (12%)	
School	1084 (94%)	69 (6.0%)	
Monastery	605 (90%)	66 (9.8%)	
Birth Location			**<0.001**
India	1366 (93%)	100 (6.8%)	
Tibet	983 (88%)	139 (12%)	
Nepal	120 (98%)	3 (2.4%)	
Unknown	53 (91%)	5 (8.8%)	
HBV Vaccine			**<0.001**
Yes	1533 (97%)	56 (3.5%)	
No	605 (78%)	169 (22%)	
Unknown	384 (95%)	22 (5.4%)	
Family History of HBV (household only)			**<0.001**
Yes	188 (81%)	45 (19%)	
No	645 (91%)	67 (9.4%)	
Other Vaccinations			0.9
Yes	1663 (91%)	166 (9.1%)	
No	804 (91%)	79 (9.0%)	
Ever any Injections			**0.04**
Yes	1521 (90%)	167 (9.9%)	
No	942 (92%)	77 (7.6%)	
History of Invasive Medical Procedure			0.1
Yes	574 (90%)	66 (10%)	
No	1892 (91%)	179 (8.6%)	
History of Blood Transfusion			0.2
Yes	71 (87%)	11 (13%)	
No	2398 (91%)	234 (8.9%)	
Tattoo			0.4
Yes	120 (93%)	9 (7.0%)	
No	2346 (91%)	235 (9.1%)	

CHAPTER 3:

Ccr5 deficiency enhances hepatic innate immune cell recruitment and inflammation in a murine model of acute hepatitis B infection.

This work has been submitted for publication and is under review:
Stevens KE, Thio CL, Osburn WO. (2018) ***Ccr5*** **deficiency enhances hepatic innate immune cell recruitment and inflammation in a murine model of acute hepatitis B infection.**

3.1 Abstract

Human genetic studies demonstrate a link between the 32-bp deletion that produces a nonfunctional CCR5 receptor (CCR5Δ32) and enhanced recovery from acute hepatitis B virus (HBV) infection. To investigate the role of CCR5 in immune responses to acute HBV, we intravenously infected *Ccr5*$^{+/+}$ (WT) and *Ccr5*$^{-/-}$ (KO) mice with a replication-incompetent adenovirus containing the overlapping HBV1.3 construct (AdHBV), or vector control. At day 3 following AdHBV infection, analysis of intrahepatic leukocytes (IHL) showed KO mice had increased CD11b^{+} NK cells compared to WT (18.2% vs 7.6% of live IHL, $p < 0.01$). These CD11b^{+} NK cells were non-resident (CD49a^{-}) and had capacity to degranulate and produce IFN-γ following stimulation. At day 3, plasma CXCL10 was significantly increased in KO, but not WT, mice receiving AdHBV as compared to vector control, while CXCR3 expression on hepatic CD11b^{+} NK cells in AdHBV-treated KO mice was significantly lower compared to uninfected mice, suggesting these NK cells are recruited along the CXCL10/CXCR3 axis. At days 7 and 14, no differences between genotypes were observed in number, or HBV-specific function, of intrahepatic CD8^{+} T cells. Instead, at day 14, KO mice had increased intrahepatic pro-inflammatory monocytes compared to WT mice (17.56% vs 6.57% of live IHL, $p = 0.014$), corresponding with an increase in plasma ALT and intrahepatic IL-1β observed in KO mice. Taken together, these findings demonstrate that loss of CCR5 signaling drives a more robust inflammatory liver microenvironment early in acute HBV infection via enrichment of hepatic innate immune cells.

3.2 Introduction

Following an acute HBV infection, the majority of infected adults mount an immune response that results in recovery, which is serologically identified by antibodies against the hepatitis B surface antigen (HBsAg). In addition to an effective antibody response, T cells and NK cells are important in control of HBV (reviewed by Schuch et al, 2014[263]). Although the mechanisms underlying the ability of some individuals to mount an effective immune response are not fully elucidated, certain host genetic factors are associated with recovery. In one study, individuals who were infected with HBV and were homozygous for a 32-base pair deletion in the CCR5 chemokine receptor (CCR5Δ32) had significantly decreased odds of developing CHB as compared to individuals homozygous for wild-type CCR5.[236] Interestingly, those who were heterozygous for CCR5Δ32 had an intermediate risk of developing persistent infection, suggesting a CCR5Δ32 gene dose effect in control of acute HBV infection.

CCR5, a chemokine receptor expressed on the surface of T cells, NK cells and macrophages, mediates immune cell trafficking into sites of inflammation by binding to its cognate ligands CCL3, CCL4, and CCL5. CCR5 binding has been shown to play a critical role in migration of memory CD8+ T cell during viral infections and regulatory T cells in cancer[264–267], but less is known about the role of CCR5 in recruitment of immune cells to the liver. In a model of autoimmune hepatitis, *Ccr5* knock-out (KO) mice exhibited more severe hepatitis due to increased hepatic recruitment of NK cells dependent upon the CCL5/CCR1 axis compared to *Ccr5* wild type (WT) mice[239,240].

In a hydrodynamic injection murine model of acute HBV, hepatic non-resident NK cells contributed to lower viral levels and an enhanced HBV-specific CD8+T cell response in an IFN-γ dependent mechanism.[147] Another study using a similar murine model found that an TLR3 signaling in addition to hepatic HBV antigen expression is required to recruit NK cells in large numbers to the liver.[145] Thus, it is intriguing to consider that decreased CCR5 may also affect NK cell recruitment in the context of acute HBV.

We used an adenoviral mouse model of acute HBV and *Ccr5* $^{-/-}$ (KO) mice to investigate the contribution of CCR5 signaling in immune cell recruitment to the liver during acute HBV infection. We found that loss of CCR5 signaling drives a robust infiltration of NK cells into the liver early in acute HBV infection. This NK cell population is likely recruited via the CXCL10/CXCR3 axis and is characterized by a significantly enhanced degranulation capacity and limited capacity to produce cytokines. In addition, later in the acute infection period, this loss of CCR5 signaling leads to an expanded pro-inflammatory monocyte population in the liver, which correlates with a significant increase in markers of hepatic inflammation. Taken together, these findings suggest that the absence of CCR5 signaling enhances immune cell migration and recruitment via alternative chemokine signaling pathways and leads to a greater pro-inflammatory response in acute HBV infection in mice.

3.3 Materials and Methods

3.3.1 Generation of HBV-expressing adenovirus

Replication-incompetent serotype 5 adenoviral vectors with (AdHBV) and without (vector control) an inserted 1.3 overlapping HBV genome cloned from HBV transgenic mice (mice courtesy of F. Chisari, lineage 1.3.32, HBV genotype D)[94] were created using the AdEasy system (Agilent Technologies, Santa Clara, CA), according to manufacturer's instructions. Both AdHBV and vector control viruses were propagated in the Ad293 cell line (Agilent Technologies, Santa Clara, CA), and titered in Ad293 cells by assessing GFP expression by flow cytometry 24 hr post-infection, as previously described.[100]

3.3.2 Murine Infection with AdHBV

All mice were handled and housed according to the guidelines set forth by the Johns Hopkins University Animal Care and Use Committee in compliance with the US National Research Council's Guide for the Care and Use of Laboratory Animals, the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use of Laboratory Animals with standard lab chow and cage enrichment. Male *Ccr5 +/+* (WT) or *Ccr5 -/-* (KO) mice (C57BL/6 background) aged 8-12 weeks were administered AdHBV or vector control via tail-vein injection ($1x10^8$ I.U., i.v.). Mice were euthanized by CO_2 asphyxiation at days 1, 2, 3, 7, and 14 post-infection (Figure 3.1a). Blood was collected by cardiac puncture and mixed with a 10% acid-citrate dextrose solution to prevent coagulation. Plasma was separated by microcentrifugation

(6000rpm, 5 minutes) and frozen at -80°C. ALT testing was performed by the Phenotyping and Pathology Core, Johns Hopkins Medical Institute.

Livers were perfused with 5 ml Kregs Ringer Buffer (KRB, 25 mM $NaHCO_3$, pH 7.4 containing 154 mM NaCl, 5.6 mM KCl, 5.5 mM Glucose, and 20.1 mM HEPES) prior to isolation of intrahepatic leukocytes (IHL). A section of liver was also flash-frozen in liquid nitrogen. In a separate experiment, spleens were collected from *Ccr5* KO mice, aged 8-10 weeks, for splenocyte isolation.

3.3.3 Isolation of Intrahepatic leukocytes and splenocytes

IHL were isolated by collagenase digestion for 45 min at 37°C in 5 ml of pre-warmed KRB buffer supplemented with 2 mM $CaCl_2$, 2 mM $MgCl_2$, 500 CDU/ml Type 4 collagenase (Worthington Biochemical, Lakewood, NJ), and 100 U/ml DNAse I (New England Biolabs, Ipswich, MA). The digestion reaction was halted by addition of ice-cold PBS supplemented with 0.5% BSA and digested liver sections were passed through a 100 mM cell strainer. Hepatocytes were removed by centrifuging twice at 50 x *g* for 5 min and supernatants were further centrifuged at 450 x *g* for 5 min to collect IHL. Red blood cells were lysed by incubation in ammonium-calcium-phosphate lysis buffer for 5 min, followed by addition of RPMI +5% FBS, after which IHL were passed through a 40 uM filter and pelleted by centrifuging at 450 x *g* for 5 min.

Splenocytes were isolated by passing disrupted spleens in RPMI media over a 100 mM filter. Cells were pelleted at 450 x *g* for 5 min and red blood cells were lysed by incubation with ammonium-calcium-phosphate buffer as described above. Followed by

addition of RPMI + 5% FBS, the preparation was passed through a 40 mM filter, and splenocytes were pelleted by centrifuging at 450 x *g*, 5 min.

3.3.4 Immunophenotyping by Flow Cytometry

IHL were stained immediately *ex vivo* with a comprehensive phenotyping panel: LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (ThermoFisher, Waltham, MA), and antibodies against CD3-PeCy5 (145-2C11, Cat#15-0031-82), CD25-PeCy7 (PC61.5, Cat#25-0251-81), CD11b- eFluor450 (M1/70, Cat#48-0112-82) from ThermoFisher (Waltham, MA); CD4-AF700 (RM4-5, Cat#100536), CD8-BV650 (53-6.7, Cat#100742), F4/80-PE (BM8, Cat#123110), Ly6c-BV570 (HK1.4, Cat#128030), and Ly6g-BV605 (1A8, Cat#127639) from Biolegend (San Diego, CA); NK1.1-PECF594 (PK136, Cat#562864) and CD19-BV711 (1D3, Cat#563157) from BD Biosciences (Franklin Lakes, NJ); and CD11c-APC (N418, Cat#130-102-493) from Miltenyi (Auburn, CA). Flow cytometry for all samples was acquired on a BD LSRII flow cytometer using FACS Diva Software (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo v. 9.9.6 (FlowJo, LLC, Ashland, OR). Flow cytometry gating schemes are available in Figure 3.2.

3.3.5 Functional Analysis of T cell populations

Following isolation, IHL were rested for 5 hours in RPMI media containing 10% FBS, 1% Penicillin-Streptomycin. To assess HBV-specific T cell responses, IHL were stimulated for 12 hours with a peptide encoding the H-2Kb restricted HBsAg epitope (2 µg/ml, ILSPFLPLL, Proimmune, Oxford, UK) and overlapping peptides spanning the full length of

the hepatitis B surface and core proteins (2 μg/ml per peptide, 15 amino acids length w/ 10 amino acids overlap, genotype D, Proimmune, Oxford, UK) in the presence of brefeldin A and monensin (GolgiPlug and GolgiStop, respectively, 1:1000 dilution each, BD Biosciences, Franklin Lakes, NJ). IHL were also stimulated for 12 hours with anti-CD3/anti-CD28 (BD Biosciences, San Jose, CA) as a positive control, or unstimulated in media alone, as a negative control. To assay cytotoxicity, anti-CD107a AF488 (1D4B, Cat#121608, Biolegend, San Diego, CA) antibody was added during the incubation. Surface staining was performed using antibodies against CD3 PeCy5, CD8+BV655, and CD4+AF700. For assessment of cytokine production, intracellular cytokine staining (ICS) was performed with anti-TNF-α-BV421 (MP6-XT22, Cat#506328, Biolegend, San Diego, CA) and anti-IFN-γ PE (XMG1.2, Cat#505808, ThermoFisher, Waltham, MA) antibodies following fixation and permeabilization, performed according to manufacturer's instructions with the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Waltham, MA**).** Flow cytometry was acquired on a BD LSRII flow cytometer using FACS Diva Software, and data analyzed using FlowJo v. 9.9.6.

3.3.6 Functional Analysis of NK Cell subpopulations

Splenocytes were isolated from untreated male 8-10 week-old *Ccr5* KO mice and incubated for 5 hours in RPMI media containing rIL-2 and rIL-15 (100 pg/ml and 1ng/ml, respectively, R&D Biosystems, Minneapolis, MN). Splenocytes (1×10^6 cells/condition) were then incubated with either 50 ng/ml PMA (phorbol 12-myristate-13-acetate) and 0.5 μM ionomycin; Yac-1 cells (ATCC, Manassas, VA), a murine lymphoma cell line that

lacks surface MHC I expression (Effector:Target ratio, 200 splenocytes: 1 Yac-1 cell); or media alone for 3 hours in the presence of brefeldin A and monensin (GolgiPlug and GolgiStop, respectively, 1:1000 dilution each, BD Biosciences). To assay degranulation potential, anti-CD107a AF488 was added during incubation. Surface staining was performed using NK1.1 PECF594, CD11b BV605 (M1/70, Cat#101257, Biolegend,San Diego, CA), CD3 PeCy5, CD8+BV655, and CD49a BV786 (Ha31/8, Cat#740919, BD Biosciences, Franklin Lakes, NJ) For assessment of cytokine production, ICS was performed, as described above.

3.3.7 Tissue preparation and quantification of liver proteins

Liver tissue (20-40 mg) was homogenized in ice-cold Tris lysis buffer (20 mM Tris, pH 7.5 containing 150 mM NaCl , 1 mM EDTA, and 1% Triton-X) supplemented with complete Mini Protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) by repeated passage through a 20 gauge needle followed by pulse sonication for 10 min. Protein isolates were assayed immediately or aliquoted and stored at -80°C. Total protein concentration was determined using the Pierce Micro-BCA protein assay kit (ThermoFisher, Waltham, MA).

Liver HBsAg was quantified by ELISA using a modified protocol for the ETI-MAK-4 HBsAg kit (Diasorin, Vercelli, Italy). In brief, the assay was performed according to manufacturer's protocol, with a standard curve (20pg-100ng, 4-fold dilution) established from recombinant HBsAg (subtype adw, Cat# ab91276, Abcam, Cambridge, UK). Liver HBsAg was normalized to total liver protein. The murine V-plex pro-inflammatory kit

from MSD was utilized to assess IFN-γ, IL-10, IL-12p70, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, and TNFα in liver specimens (Mesoscale Discovery, Rockville, MD). Cytokine concentrations were normalized to liver total protein levels.

3.3.8 Quantification of Plasma HBV DNA

HBV DNA was purified from 100 µl Plasma by Qiagen MinElute Virus Spin Kit (Qiagen, Hilden, Germany) per manufacturer's instructions, and eluted in 40 µl H_2O. HBV DNA was quantified by SYBR green assay utilizing the HBV specific primers from Integrated DNA Technologies ((IDT, San Jose, CA):

HBV1745f28 (5'- GTTGCCCGTTTGTCCTCTAATTCCAGG-3')

HBV2144r29 (5' GGCCCCCAATACCACATCATCCATATAAC-3')

3.3.9 Plasma quantification of chemokines

The murine chemokine U-plex kit from Meso Scale Discovery (Rockville, MD) was utilized to assess IP-10, KC/GRO, MCP-1, MIP-1α, MIP-1β, MIP-2, and MIP-3α in 25 mL of plasma collected from AdHBV or vector control treated mice at indicated time points according to manufacturer's instructions.

3.3.10 Statistical Analysis

To analyze the differences between groups, a two-tailed Wilcoxon Rank Sum test was utilized with $p < 0.05$ considered statistically significant. Data are expressed as mean +/- SD unless otherwise noted.

3.4 Results

3.4.1 Increased CD11b+ NK Cells observed at Day 3 in KO mice following AdHBV injection

In both WT and KO mice, AdHBV injection resulted in peak hepatic HBsAg levels on days 3 and 7 (Figure 3.1B) and peak plasma HBV DNA at day 3 (Figure 3.1C). At this day 3 peak, the proportion of live IHL that were $CD11b^+$ $NK1.1^+$ NK cells was significantly higher in KO compared to WT mice following AdHBV infection (18.2% vs. 7.6% of live IHL, $p < 0.01$, Figure 3.3A and 3.3B). These intrahepatic $CD11b^+$ NK cells were predominantly $CD49a^-$, consistent with an infiltrating NK cell population[268] (representative plot, Figure 3.4B). The proportion of the other NK cell types, $CD11b^-$ $NK1.1^+$ and $CD3^+NK1.1^+$ NK T cells, did not differ by *Ccr5* genotype (Figure 3.4A). Further, there were no differences in proportions of other live IHLs at day 3 by genotype (Figure 3.4C). These data suggest that *Ccr5* deficiency is associated with increased levels of infiltrating, not resident, NK cells early in acute HBV infection.

3.4.2 Increased plasma IP-10 and decreased surface expression of CXCR3 on CD11b+ NK cells at day 3 in KO mice

To determine whether these $CD11b^+$ NK cells were migrating into the liver along the CCL5/CCR1 axis, as observed in the autoimmune hepatitis model[8] , or by a different chemokine/receptor pathway, chemokines were measured in the plasma and livers of mice following exposure to AdHBV or control vector. At day 3, plasma levels of the CXCR3 ligand IP-10 were significantly higher in KO compared to WT mice (3.0 vs 1.2

fold-increase over vector control-treated mice, $p<0.05$, Figure 3.3C). At this time, surface expression of CXCR3 was significantly decreased on $CD11b^+$ NK cells in the liver, but not spleen, of KO mice (% $CXCR3^+$ in $CD11b^+$ NK cells, 18.7 vs. 35.7 at day 0, $p = 0.03$, Figure 3.3D), suggesting CXCR3 is recycled from the surface of $CD11b^+$ NK cells following earlier receptor engagement in recruitment to the liver.

Plasma levels of the CXCR2 ligands CXCL1 and CXCL2 were also significantly higher at day 3 in KO compared to WT mice (CXCL1: 4.9 vs. 1.4 and CXCL2: 3.7, $p < 0.05$, Figure 3.5A). However, consistent with its role in neutrophil recruitment, CXCR2 was not detectable on the surface of $CD11b^-$ or $CD11b^+$ NK cells in the spleen or livers of KO mice (data not shown). Unlike the autoimmune hepatitis model, no significant differences in the three CCR5 ligands, CCL3, CCL4, and CCL5, were observed in the plasma between KO and WT mice relative to the levels in the respective KO and WT vector control treated mice (Figures 3.5B and 3.6). Further, unlike the ConA model of autoimmune hepatitis, liver CCL5 expression was not increased at day 3 following AdHBV infection (Figure 3.5C). Taken together, these findings suggest that IP-10/CXCR3 signaling may be driving recruitment of $CD11b^+$ NK cells into the liver.

3.4.3 Increased degranulation potential of intrahepatic CD11b+ NK Cells

To examine the effector capacity of the $CD11b^+$ infiltrating NK cell population, we assessed the degranulation and cytokine production potential of $CD11b^+$ and $CD11b^-$ splenic NK cell populations from KO mice. In response to PMA/ionomycin or Yac1 stimulation, $CD11b^+$ NK cells had increased degranulation potential, represented by

increase cell surface levels of CD107α, compared to unstimulated cells (21.7 vs. 3.7 vs. 1.5, $p = 0.027$, Figure 3.7A), while this was not observed in the $CD11b^-$ NK cell population. Both $CD11b^+$ and $CD11b^-$ NK cells produced significantly more IFN-γ in response to PMA/ionomycin stimulation compared to no stimulation ($IFN\text{-}\gamma^+$ $CD11b^+$ cells, 8.85% vs. 1.10%, $p < 0.05$; $IFN\text{-}\gamma^+$ $CD11b^-$ cells 20.66% vs. 4.21%, $p < 0.05$, Figure 3.7B). These data demonstrate that $CD11b^+$ NK cells have both degranulation potential and cytokine-production capacity.

3.4.4 Increased markers of inflammation in KO mice at Day 14 following AdHBV infection

To assess whether the early-phase infiltrating NK cell population affected the inflammatory response later in acute HBV, we measured ALT activity in plasma samples obtained on days 1, 2, 3, 7, and 14. At day 14, KO mice had significantly increased ALT compared to WT mice relative to the levels observed in vector control-treated mice (129% vs. 67% of control mice respectively, $p < 0.01$, Figures 3.8A and Figure 3.9A), but no differences were observed by genotype at earlier time points.

To further explore the inflammatory response at day 14, we assayed pro-inflammatory cytokines in liver tissue. Both IFN-γ (0.91- vs 0.47-fold over vector control-treated mice, $p < 0.05$) and IL-1β (1.86 vs 0.65 fold over control mice, $p < 0.05$) were significantly increased at day 14 in KO compared to WT mice relative to the levels in vector control-treated mice (Figure 3.8B). Interestingly, IL-18 levels in liver homogenate did not differ

between genotypes or between vector-control treated and HBV-treated mice (data not shown).

To assess which immune cells may be contributing to this enhanced pro-inflammatory environment in KO mice, we measured IHL populations at day 14 following injection with AdHBV or vector-control. The proportion of live IHL that were $Ly6c^{hi}CD11b^{+}$ $CD11c^{-}$ monocytes were significantly greater in AdHBV-treated KO mice compared to AdHBV-treated WT mice (17.56% vs. 6.57% of total live leukocyte population, $p = 0.014$, Figure 3.8C), while other cell populations did not differ by genotype (Figure 3.9B). There were no significant differences in the numbers of CD4+and CD8+T cell populations by genotype, and $CD8^{+}$ T cells obtained from KO and WT mice produced similarly low levels of CD107α, TNF and IFN-γ in response to stimulation with HBV-specific peptides (Figure 3.9C and 3.9D). These data suggest that the pro-inflammatory response observed in the livers of *Ccr5*-deficient mice at day 14, is due to recruitment of $Ly6c^{hi}$ monocytes into the liver.

3.5 Discussion

Using a murine model of acute HBV infection, this study demonstrates that *Ccr5* deficiency enhances recruitment of infiltrating NK cells to the liver. Correlation of this enhanced infiltration of NK cells with elevated plasma IP-10 and down-regulation of CXCR3 on the infiltrating NK cells provides evidence that the IP-10/CXCR3 axis may guide this NK cell recruitment. Further, *Ccr5* deficiency enhanced hepatic inflammation and hepatocyte damage characterized by increased ALT that coincided with enhanced

recruitment of pro-inflammatory monocytes into the livers of *Ccr5* KO mice. Taken together, these findings of altered intrahepatic immune cell composition in the absence of CCR5 may explain why individuals with the CCR5Δ32 mutation are more likely to recover from an acute HBV infection.

Several lines of evidence support a role for NK cells for recovery from acute HBV. Chimpanzee studies suggest that NK cells have a non-cytopathic role in control of acute HBV via secretion of IFN-γ and TNF prior to the arrival of T cells in the liver.[123] Mouse models of acute and chronic HBV confirm a role for NK cells in non-cytopathic control of viral replication and in shaping adaptive T cell responses, but there is inconsistency between studies regarding whether NK cells are recruited in response to HBV alone or require an external stimulus, such as TLR3 activation.[143,144,147,240] In this acute HBV model, it was found that there is an increase in NK cell recruitment to the liver early in acute infection in the absence of an external stimulus in *Ccr5* deficient compared to WT mice.

It is interesting to consider why absence of CCR5 led to enhanced NK cell recruitment, likely via the IP-10/CXCR3 axis. One potential mechanism to explain why NK cells from *Ccr5* KO mice may be more susceptible to CXCR3-mediated trafficking is chemokine receptor crosstalk and desensitization. Receptor desensitization occurs when signaling via one chemokine receptor desensitizes a different chemokine receptor by either competition for downstream signaling molecules or a direct interaction between receptors. Both CCR5 and CXCR3 are expressed on NK cells, and while these two receptors do not compete for ligands, there is evidence from FRET assays that

CXCR3 and CCR5 can form heteromers.[269] In T cells, CXCR3 agonism has been shown to result in cross-phosphorylation of CCR5 and inhibition of CCR5-mediated chemotaxis.[269] Similarly, CCR5 signaling following IL-16/CD4+stimulation was recently shown to result in desensitized CXCR3 signaling.[270] Thus, if CXCR3 signaling is the major axis for NK cell recruitment in acute HBV infection, signaling via CCR5 may attenuate this signal in *Ccr5* WT mice, thereby offering an explanation for the enhanced NK cell chemotaxis observed in *Ccr5* deficient mice.

This study also identifies a novel role for *Ccr5* deficiency in recruitment of pro-inflammatory monocytes to the liver in the later stages of acute HBV infection. In contrast to the present study, previous murine experiments have demonstrated that treatment with an adjuvant (e.g. TLR9 ligands) was essential for recruitment of large populations of pro-inflammatory monocytes into the liver in the context of HBV infection.[160,271] Following administration of TLR9 ligands, Huang et al. (2013) demonstrated that pro-inflammatory monocytes establish local inflammatory pockets within the liver called iMATES (intrahepatic myeloid-cell aggregates for T cell population expansion.[160] These iMATES facilitated T cell priming and development of functional anti-HBV T cell responses, suggesting that the increased hepatic pro-inflammatory monocytes observed in the current experiments may help shape subsequent cellular immune responses in KO mice. Zhao et al. (2017) found similar results following administration of GM-CSF during DNA vaccination in a murine model of CHB.[271] Interestingly, the requirement for adjuvant stimulation to drive pro-inflammatory monocyte recruitment to the liver was not observed in *Ccr5*-deficient mice. Further

study is warranted to determine if these pro-inflammatory monocytes establish iMATES and influence anti-HBV T cell responses later in HBV infection in the absence of functional CCR5 signaling.

The hepatic infiltration of pro-inflammatory monocytes observed in KO mice was accompanied by increased hepatic inflammation and hepatocyte damage, as characterized by increased serum ALT and liver IL-1β. IL-1β is produced by pro-inflammatory monocytes following signaling of pattern-recognition receptors; while it is often a downstream product of inflammasome activation, there was not a corresponding increase in IL-18, suggesting the increase in IL-1β may be inflammasome-independent. IL-1β is associated with increased antigen presentation and T cell priming[272,273] further suggesting that *Ccr5* deficiency may facilitate development of anti-HBV specific T cell responses that correlate with recovery, but further studies are required to elucidate the full contribution of the pro-inflammatory monocytes and IL-1β in HBV infection

While this study is limited by the nature of the model as a single round of HBV infection, the use of a murine model permits analysis of the hepatic anti-HBV immune responses at multiple time points and across multiple *Ccr5* genetic backgrounds, thereby facilitating a better understanding of the immune contribution of CCR5 in the liver following acute HBV infection. Both NK cells and pro-inflammatory monocytes have been shown to enhance adaptive responses in the context of HBV, but only minimal functional HBV-specific CD8+T cell responses were observed in both WT or KO mice by day 14 in this study, so future experiments would be required to examine the role of

Ccr5 in development of adaptive cellular immune responses later in infection. Further, since development of HBV-specific CD8+T cell responses is dependent upon the dose of viral challenge,[92] further experimentation would be required to elucidate the full contribution of CCR5 across different HBV infection conditions.

Taken together, this study identifies a novel role for CCR5 signaling in modulation of hepatic innate immune cell populations and inflammation in the context of acute HBV infection. Loss of functional CCR5 signaling enhances hepatic recruitment of innate immune cell populations providing further evidence that targeting CCR5 signaling *in vivo* may represent a novel therapeutic opportunity to facilitate functional control of HBV infection.

Figure 3.1. Experimental design and early viral dynamics

A) Experimental design and dosing scheme. (B) Hepatic levels of HBsAg during acute HBV infection of WT and KO mice, normalized to total liver protein. (C) Plasma HBV DNA levels. Symbols represent values from one mouse. Line indicates the mean ± SD of the group.

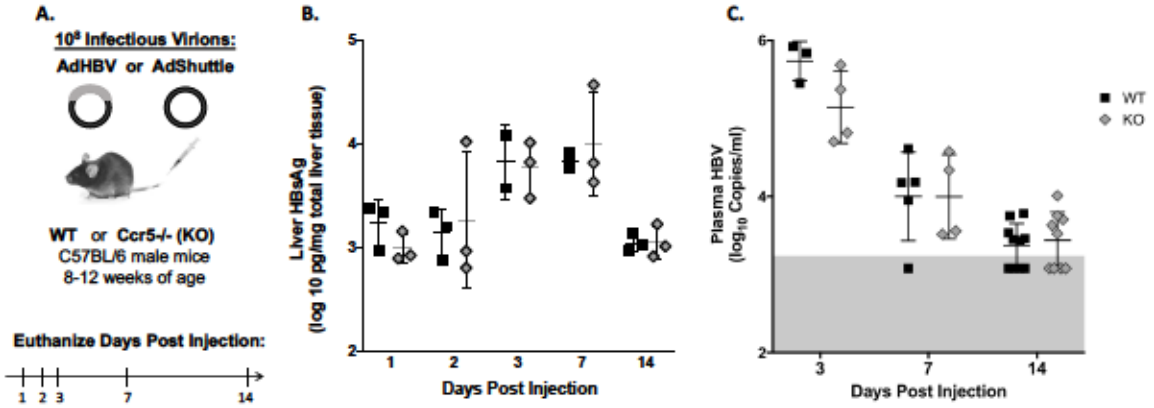


Figure 3.2 Gating Scheme for analysis of intrahepatic leukocytes (IHLs).
The total number of live leukocytes was calculated as the sum of Neutrophils, CD3+ T Cells, B Cells, NKT cells, NK Cells, CD11b+, F480+CD11b-, and CD11b-F480- cells (after subtracting out lineage negative cells).

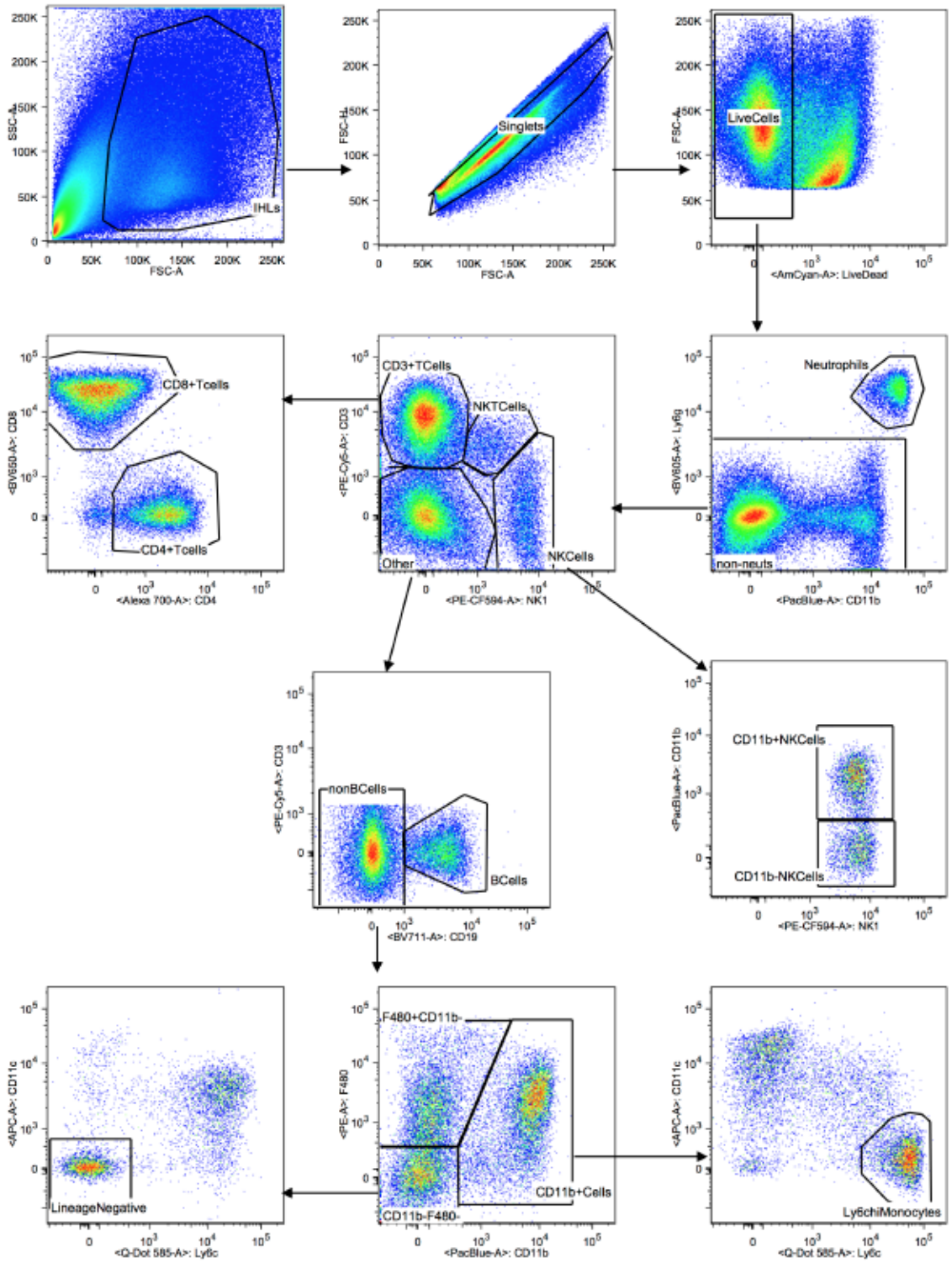


Figure 3.3. Infiltrating CD11b+ NK cells peak at Day 3 in KO mice, are associated with increased plasma IP-10, and have decreased surface expression of CXCR3

(A) Intrahepatic $CD11b^{+}NK1.1^{+}$ NK cells as a proportion of total live leukocytes in AdHBV-infected and vector control mice, assayed by flow cytometry as shown in Figure 3.2. Symbols represent values from one mouse. n=3-10 mice/group from 1-2 independent experiments per group. Line indicates the mean ± SD of the group.

(B) Representative dot plots of Intrahepatic leukocytes from KO (top) and WT (bottom) mice.

(C) Increased expression of plasma IP-10 assayed by MSD at Day 3 relative to vector-treated mice from the same genetic background. Symbols represent values from one mouse. Line indicates the mean ± SD of the group.

(D) Proportion of CD11b+ NK cells expressing CXCR3 in the spleen and liver of KO mice pre-infection (black bars) or at Day 3 post-infection (grey bars). Each line represents one mouse.

* P<0.05, ** P<0.01 using a two-tailed Wilcoxon rank test

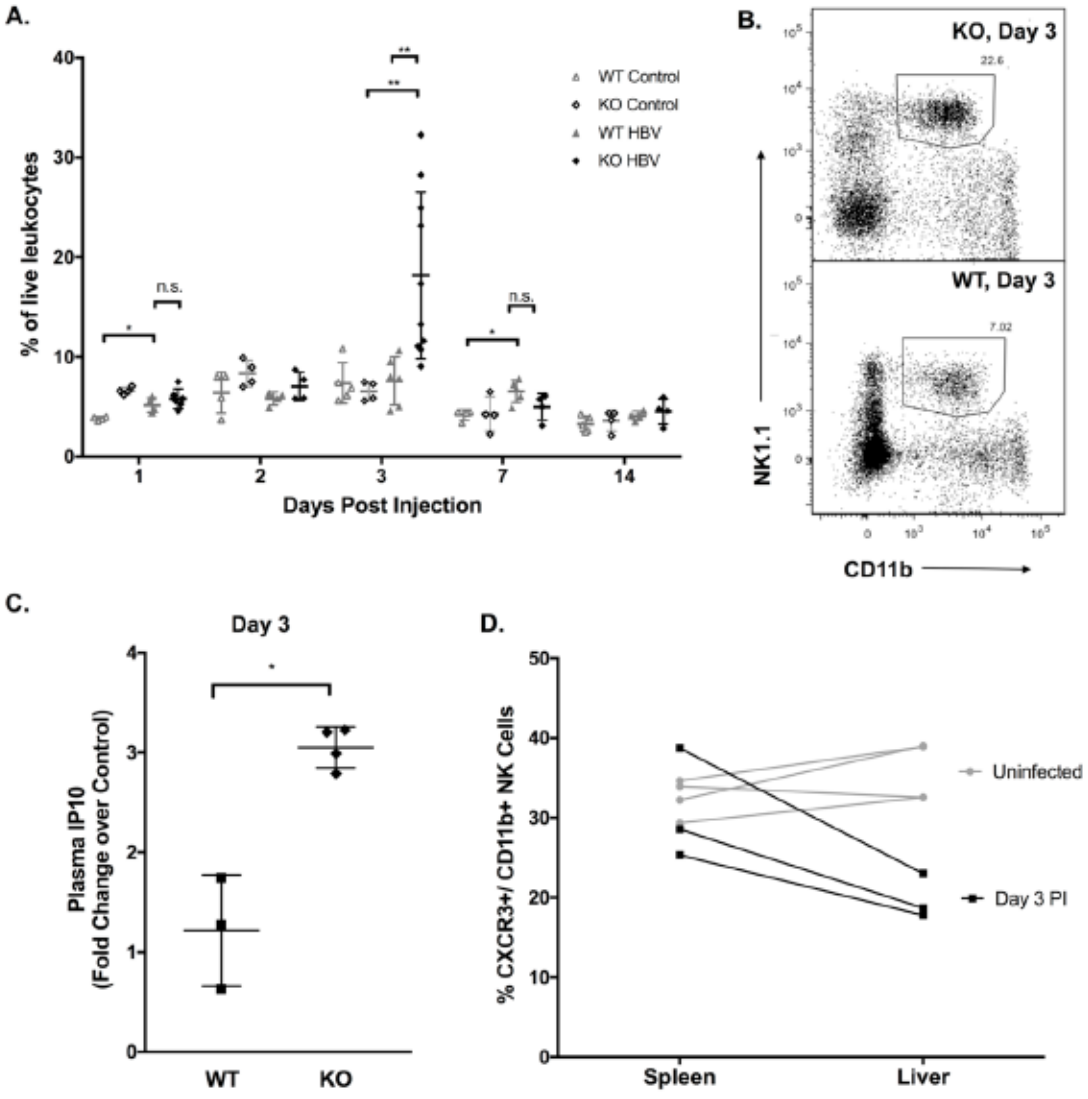


Figure 3.4. Infiltrating CD11b+ NK cells peak at Day 3 in KO mice.

(A) Intrahepatic $CD11b^{-}NK1.1^{+}$, $CD11b^{+}NK1.1^{+}$, and NKT ($CD3^{+}NK1.1^{+}$) cells at day 3 PI assessed by flow cytometry and gated as shown in Figure 3.2.

B) Representative dot plot from flow cytometry, gated on $NK1.1^{+}$ cells showing that most $CD11b^{+}$NK cells are CD49a-.

(C) Total IHL Populations at Day 3 post-infection. T cells (CD3), $CD4^{+}$ T Cells (CD4), $CD8^{+}$ T Cells (CD8), B cells (CD19), $Ly6g^{hi}CD11b^{+}$ neutrophils (Neutrophils), and $CD11b^{+}Ly6c^{hi}$ monocytes populations assessed by flow cytometry and gated as shown in figure 3.2.

Symbols represent values from one mouse. n=3-10 mice/group, 1-2 independent experiments per group. Line indicates the mean ± SD of the group. * P<0.05, ** P<0.01 using a two-tailed Wilcoxon rank test.

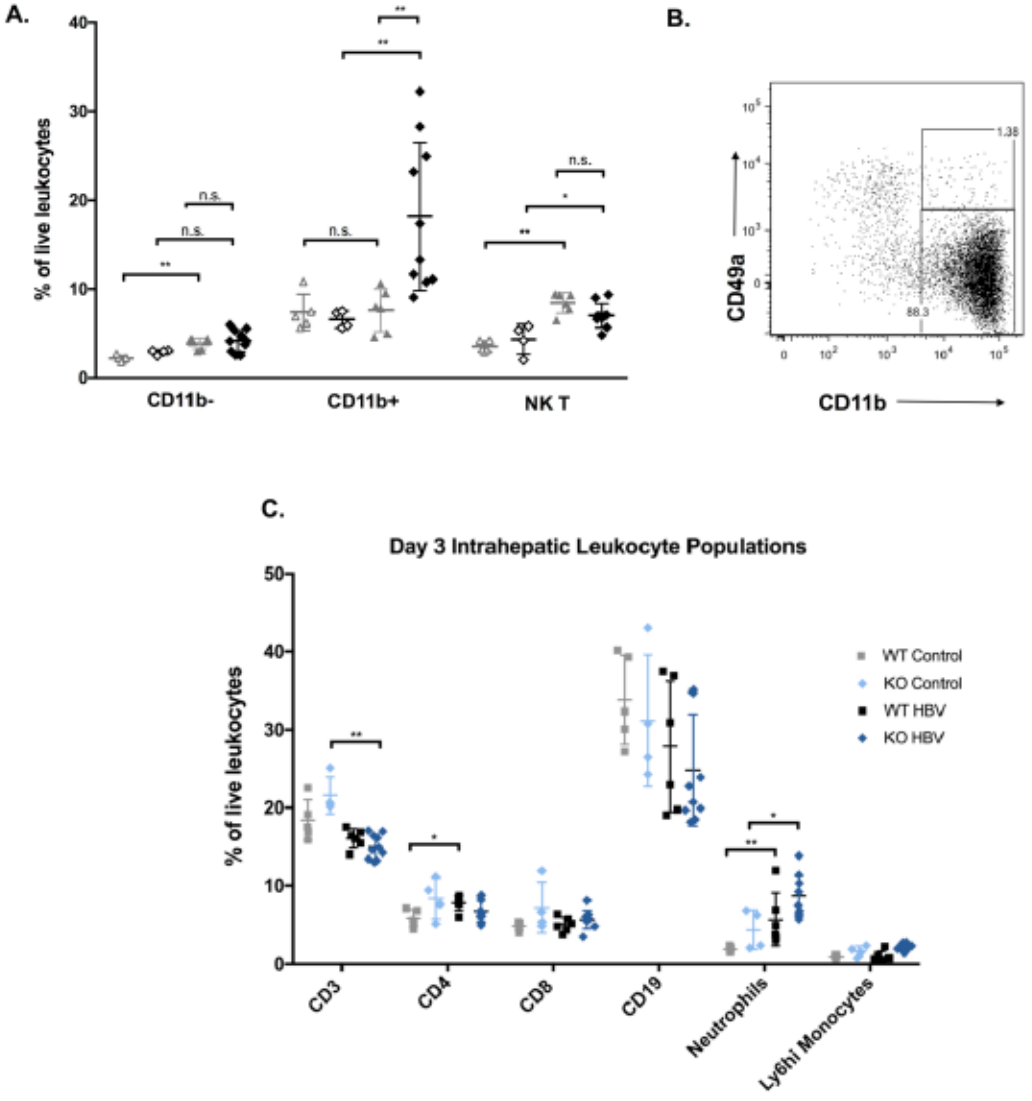


Figure 3.5. Increased expression of plasma CXCR2, but not CCR5, ligands in KO mice
(A) Plasma CXCR1 and CXCR2 or (B) CCL3, CCL4, and CCL5 at Day 3 relative to vector-treated mice from the same genetic background. (C) Liver CCL5 assayed at Day 3 in KO and WT mice following treatment with Control or HBV vector. Symbols represent values from one mouse. n=3-4 mice/group. Line indicates the mean ± SD of the group. * P<0.05 using a two-tailed Wilcoxon rank test.

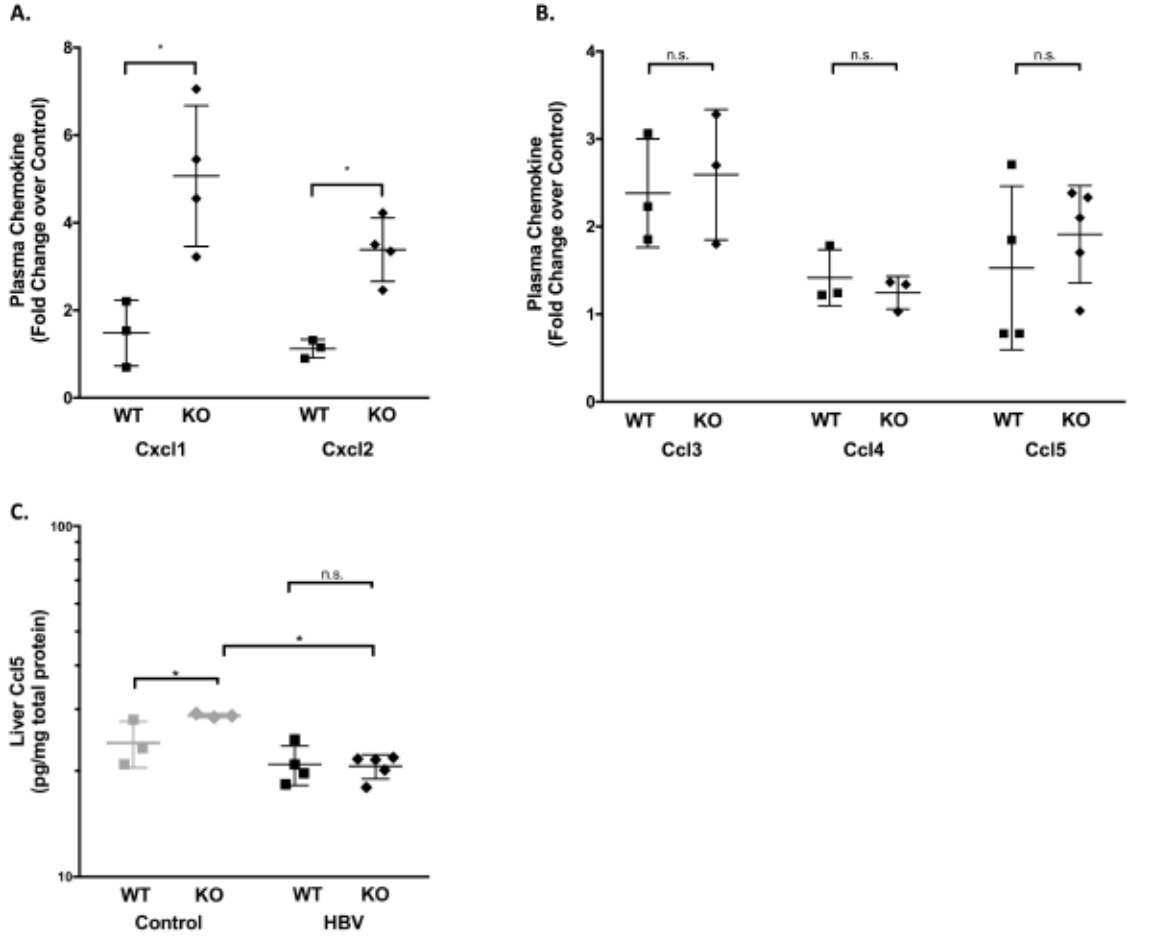


Figure 3.6. Plasma CCR5 ligands at Day 3

Total concentrations of plasma CCL3 and CCL4 assayed by MSD assay, and CCL5 assayed by ELISA. Symbols represent values from one mouse. n=3-5 mice/ group, from 1-2 independent experiments per group. Line indicates the mean ± SD of the group. * P<0.05 using a two-tailed Wilcoxon rank test.

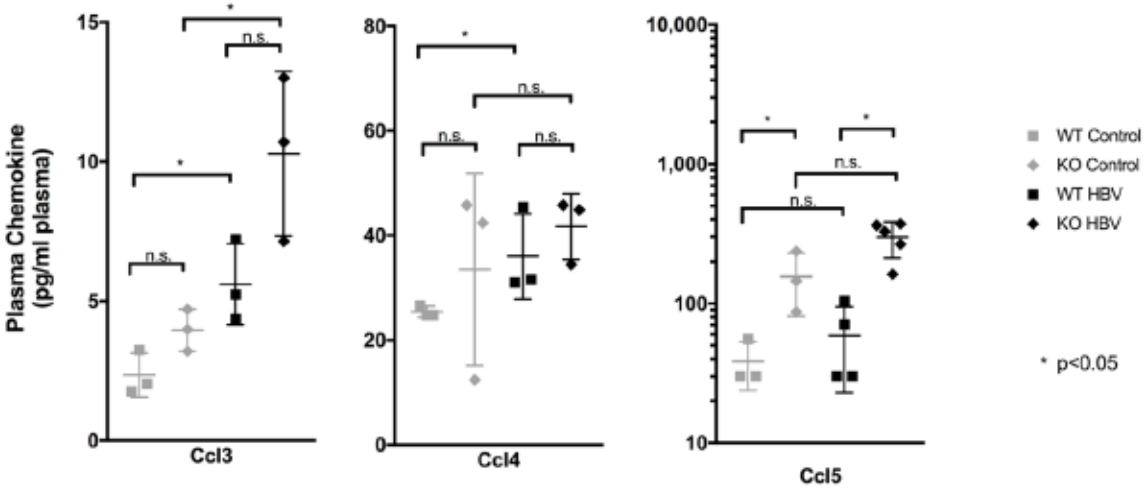


Figure 3.7. CD11b+ NK cells have cytotoxic and cytokine-producing capacity.

Characterization of NK cells from KO mice following stimulation of splenocytes with PMA/Ionomycin (50 ng/ml PMA and 0.5 µM ionomycin) or Yac1 (Effector (splenocytes):Target cell Ratio, 200:1). Function assessed as (A) degranulation potential measured by CD107α production or (B) IFNγ production. Symbols represent values from one mouse. n=3 mice. Line indicates the mean ± SD of the group. * P<0.05, ** P<0.01 using a two-tailed Wilcoxon rank test.

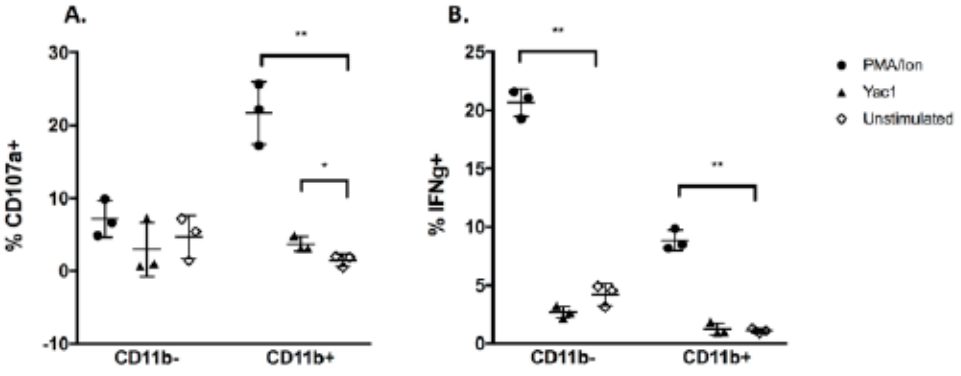


Figure 3.8. Enhanced hepatic inflammation and Ly6c^{hi} monocytes at Day 14 in KO mice.
(A) Increased ALT in KO mice.
(B) Increased hepatic levels of IFNγ and IL- 1β in KO mice.
(C) Increased pro-inflammatory Ly6c^{hi} monocytes in livers of KO mice.
(D) Representative dot plots showing Ly6c expression on CD11b^{+} monocytes
Symbols represent values from one mouse. n=3-6 mice/ group. Line indicates the mean ± SD of the group. * P<0.05, ** P<0.01 using a two-tailed Wilcoxon rank test

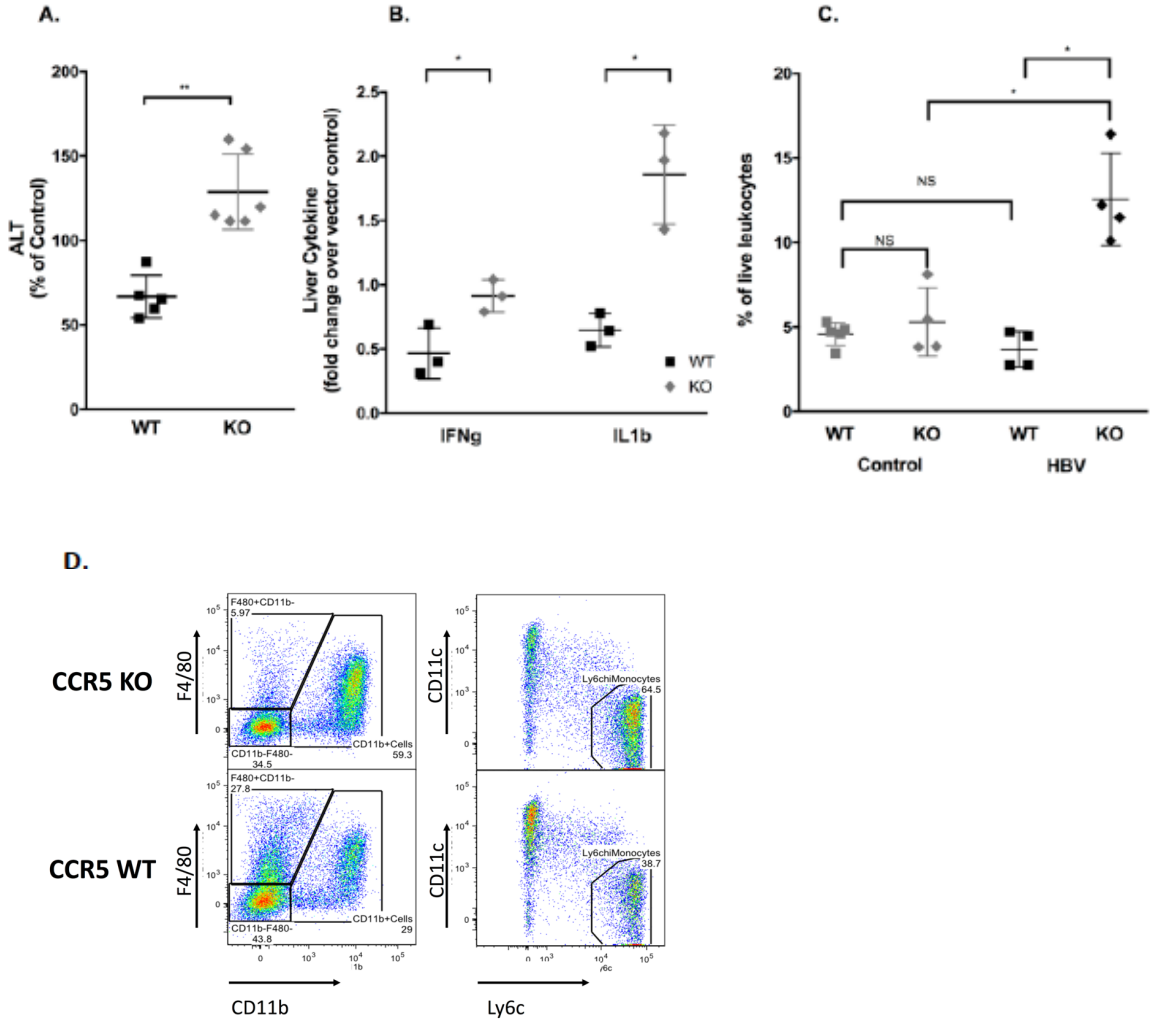


Figure 3.9. Other immune cells not altered at Day 14 in CCR5 KO mice

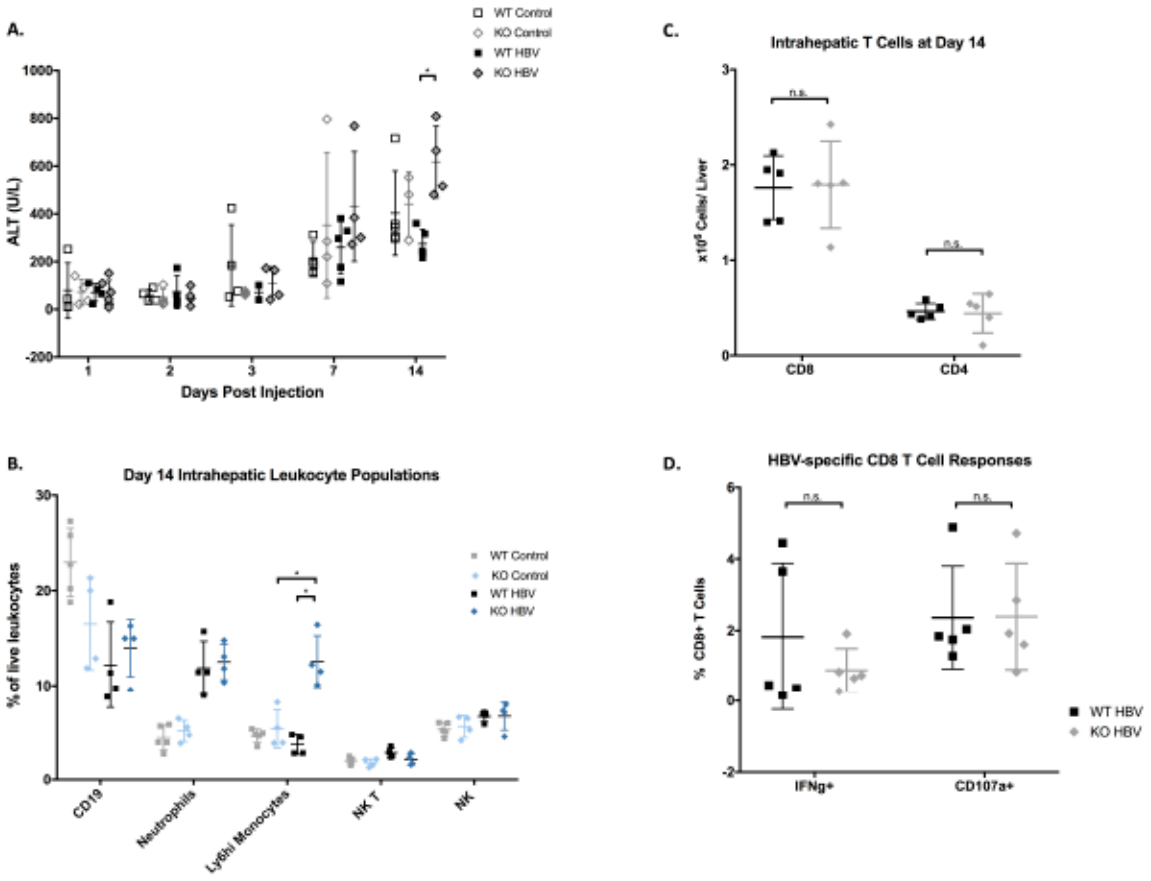
(A) Plasma ALT by days post-injection.

(B) Proportion of live leukocytes that are T cells (CD3), $CD4^+$ T Cells (CD4), $CD8^+$ T Cells (CD8), B cells (CD19), $Ly6g^{hi}CD11b^+$ neutrophils (Neutrophils), and $CD11b^+Ly6c^{hi}$ monocytes at day 14 post-infection, detected by flow cytometry and gated as shown in Figure 3.2.

(C) Total number of intrahepatic T Cells determined by Trucount beads at Day 14 in WT and KO mice following AdHBV infection.

(D) Cytokine production (IFNγ+) and degranulation potential (CD107a+) of day 14 intrahepatic CD8+ T cells following HBV-peptide stimulation for 12 hours in the presence of brefeldin-A and monensin.

Symbols represent values from one mouse. Line indicates the mean ± SD of the group. * P<0.05 using a two-tailed Wilcoxon rank test.



CHAPTER 4:

Peripheral Cytokines and Chemokines as Markers of Immune Activation in Acute HBV Infection

4.1 Introduction

Cytokines and chemokines play a major role in control of immune function to infections, and their systemic detection can serve as biomarkers of different stages or severity of infection. To date, acute hepatitis B infection has been characterized by increases in peripheral pro-inflammatory and Th1-associated cytokines, including IL-2, IL-6, IL-15, TNF, and IFNγ[180,199,206]. These findings are consistent with the known role of Th1 T cells in non-cytopathic control of HBV in animal models[97,123]. Chronic, or persistent, HBV, on the other hand, is characterized by decreased peripheral IL-2 and IFNγ, further suggesting these cytokines may play a role in recovery[199,207]. HBeAg seroconversion in those with CHB is associated with increased serum levels of IL-18 and IP-10, and decreased levels of IL-6[177,274,275]. Interestingly, the canonical anti-inflammatory cytokine IL-10 is elevated in both acute self-limited HBV and persistent HBV infection as compared to healthy controls; it is further elevated following HBeAg seroconversion in persistent infection, suggesting that it might exert pleiotropic effects at different stages of infection[206,220,224].

It remains unclear whether individuals who develop persistent HBV infection have similar cytokine profiles as those that recover during acute infection, and only later develop altered immune profiles as a result of chronic antigen exposure; or, alternatively, if individuals who develop persistent infection have different cytokine profiles during acute infection that may suggest an immune pathway responsible for development of persistent HBV infection.

To fill this gap in knowledge, peripheral cytokines and chemokines were measured before, during, and ~1 year following incident HBV infection in 173 men (n=62, 36% with HIV-infection) enrolled in the Multi-center AIDS Cohort (MACS). Of these men, 161 (93%) recovered from acute HBV infection; ten HIV-uninfected (9% of HIV-uninfected) and twelve HIV-infected (19% of HIV-infected) individuals developed persistent HBV infection. It was hypothesized that cytokines associated with Th1 responses (TNF, IL-12, IFNγ), innate control of viral hepatitis (IL-18), and interferon-stimulated genes (IP-10) would be elevated in individuals who recover from acute HBV infection as compared to individuals who develop persistent HBV.

4.2 Materials and Methods

4.2.1 Study Population

This study was conducted in a nested cohort of acutely HBV-infected individuals within MACS, a prospective cohort study of men who have sex with men (MSM) from Baltimore/ Washington DC, Chicago, Los Angeles, or Pittsburgh. From the 6,792 individuals enrolled in the MACS study since 1984, 180 individuals were identified as having incident HBV infection by testing for HBsAg, anti-HBc, and anti-HBs. Incident HBV infection was defined as individuals who were negative for all three markers and then became either HBsAg+/ anti-HBc-, HBsAg+/anti-HBc+ or HBsAg-/ anti-HBc+. Individuals were not included if they had previous exposure to HBV (defined as anti-HBc+ at baseline) or a history of hepatitis C infection (defined as positive for antibodies against HCV by EIA and HCV RNA+ at one of two visits prior to acute HBV infection).

Individuals were excluded if they had incident coinfection with HIV and HBV. The final sample size for inclusion was 173 individuals, of which 110 (63.6%) were HIV-uninfected and 63 (36.4%) were HIV-infected at baseline.

HBV recovery was defined by loss of circulating HBsAg, with or without the development of antibodies against HBsAg (anti-HBs) at 10-20 months following incident HBV infection. HBV persistence was defined serologically as HBsAg-positive at 10-20 months following incident HBV infection.

4.2.2 Sample Collection

Serum and plasma samples were collected every six-months and stored at -80°C. To limit freeze-thaw cycles, samples were aliquoted prior to testing and re-freezing. When possible, samples were tested from three time points for each participant: Pre-infection baseline (visit 1), acute Infection (visit 2), and follow-up (visit 3) at 6-18 months following visit 2 (study design outlined in Figure 4.1). The estimated time of incident HBV infection was calculated as the midpoint between the last HBsAg-/anti-HBc- pre-infection visit (visit 1) and the first acute visit (visit 2). Median time from estimated incident HBV infection for visits 1, 2, and 3, respectively, were -0.25 years (IQR: -0.28-0.25), 0.25 years (IQR: 0.25-0.28), and 1.2 years (IQR: 1.08-1.26) (Figure 4.2). Of the 173 participants, 159 (91.9%) had samples available for all three time-points (Table 4.1).

4.2.3 Serology Testing

Detection of HBsAg, anti-HBc, and anti-HBs were performed by ELISA according to manufacturer's instructions (Diasorin, Saluggia, IT). Serum AST activity was measured in a spectrophotometer in a microplate-adapted protocol of the Pointe AST assay (Pointe Scientific, Canton, MI, USA). In-house AST values correlated with clinical AST values when available ($r=0.89, n=98, p<0.001$).

Cytokine tests were performed by standard ELISA or multiplexed electrochemiluminescence assay (Meso Scale Discovery, Rockville, MD) (see Table 4.2 for a list of assay type by analyte). Assays were performed according to manufacturers' instructions at the Johns Hopkins School of Medicine.

The lower limit of quantification (LLOQ) for each analyte was determined as the lowest standard at which there was less than 15% variance from the known concentration in the calculated values for each of two standard replicates. Samples that fell below the LLOQ were considered undetectable and were assigned a value equal to ½ of the analyte's LLOQ. Summaries of the number of detectable samples at each visit are provided in Table 4.3. Analytes for which >30% of samples had concentrations above the LLOQ were included for subsequent analysis (Table 4.3)

4.2.4 Statistical Analysis

As the majority of cytokine and chemokine concentrations were not normally distributed following log10 transformation, non-parametric Kruskal-Wallis and Mann-Whitney/ Wilcoxon rank sum tests were utilized to compare across and between

groups, respectively. Differences between visit 2 fold-changes (same-person visit2/visit1) were assessed by univariate analysis, and by logistic regression normalizing for age, race, and BMI.

NxN correlation matrices were produced to assess patterns of highly correlated cytokine production. Principal component analysis (PCA) was performed with mean-centered fold-change (visit2/visit1) values for analytes for which >30% detection above the LLOQ was achieved.

4.3 Results

4.3.1 Baseline Characteristics

A total of 110 HIV-uninfected and 63 HIV-infected men were included for analysis. Of the 110 HIV- uninfected individuals, ten (9.3%) developed persistent HBV infection, while twelve of the 63 HIV-infected individuals (19%) developed persistent HBV infection. Baseline age, BMI, and AST values were not significantly different between individuals with HBV recovery and HBV persistence, regardless of HIV status (Table 4.4). HIV-infected individuals (n=62) had significantly higher baseline levels of IFNγ (6.1, IQR:2.7-9.0, vs. 2.7, IQR:2.7-2.7, pg/ml, p<0.0001), IL-10 (0.4, QR:0.2-0.6, vs. 0.2, IQR:0.2-0.4, pg/ml, p<0.0001), IL-15 (2.2, IQR:1.8-2.5, vs. 2.0, IQR:1.7-2.4, pg/ml, p=0.035), IL-18 (691.4, IQR:506.9-1191.0, vs. 427.2, IQR:314.0-574.5, pg/ml, p<0.0001) TNF (2.6, IQR: 1.9-3.9, vs. 1.6, IQR: 1.2-2.0, pg/ml, p<0.0001), IP-10 (342.6, IQR: 223.9-605.3, vs. 149.9, IQR:95.6-180.7, pg/ml, p<0.0001) and MIP1β (18.5, IQR:11.8-28.6, vs. 14.7, IQR: 9.5-20.4, pg/ml, p=0.007) compared to HIV-uninfected individuals at baseline

(Table 4.5). HIV-uninfected individuals who developed persistent HBV infection had higher baseline levels of IL-15 as compared to HIV-uninfected individuals who recovered (2.4, IQR:2.1-2.7, vs. 1.9, IQR:1.7-2.4, pg/ml, p=0.32 in univariate analysis, p=0.049 adjusting for age, race and BMI), but no difference in baseline circulating IL-15 was observed between HIV-infected individuals by HBV outcome. Instead, lower baseline IL-8 was detected in HIV-infected individuals who develop persistent HBV as compared to HIV-infected individuals who recover from acute HBV infection (9.3, IQR: 7.9-11.7, vs. 14.1, IQR:9.8-24.7, pg/ml, p=0.018 in univariate analysis, p=0.058 adjusting for age, race and BMI).

4.3.2 Characterization of acute infection at visit 2

Symptomatic acute HBV infection is characterized clinically by increases in detectable liver enzymes in serum, but the majority of cases of acute HBV infection are asymptomatic[11]. AST was tested to determine if serum liver enzymes are elevated in incident HBV infection in this mostly asymptomatic cohort. While there was no significant difference between the median AST values between those with HBV recovery and HBV persistence at visit 2 (Table 4.6), the change in AST from visit 1 to visit 2 was significantly increased in HIV-uninfected individuals who developed HBV persistence versus those with HBV recovery (10.6 vs. 0.6, median fold-change over baseline, p<0.001, Figure 4.3A). By visit 3, AST values were significantly higher in individuals with HBV persistence in both HIV-infected (46.9, IQR: 30.6-93.6, vs. 22.4, IQR: 18.1-37.9,

p=0.002) and HIV-uninfected individuals (52.3, IQR: 27.9-187.3, vs. 19.3, IQR:14.4-24.8, p=0.008).

Higher AST values were associated with increased HBV DNA levels at visit 2 (r=0.36, n=34, p=0.03, Figure 4.3B). HIV-uninfected individuals with HBV recovery had lower plasma HBV DNA levels at visit two as compared to those with HBV persistence (4.07, IQR: 3.15-6.20, n=16 vs. 7.60, IQR: 7.24-7.93, n=5, log 10 copies/ml, p=0.008). No differences in HBV DNA were observed by HBV outcome in HIV-infected individuals.

4.3.3 Cytokine changes in acute HBV infection

Concentrations of circulating cytokines at visits 2 and 3 are reported in Tables 4.7 and 4.8, respectively. Fold-changes from baseline are reported in Tables 4.9 A & 4.9 B and Figure 4.4. In both the HIV-infected and HIV-uninfected populations, individuals who develop persistent HBV infection have greater increases in circulating MIP1α (HIV-uninfected: 1.54 vs. 1.08, p=0.003, HIV-infected: 1.14 vs. 1.03, p=0.005, median fold-change over baseline) at visit 2 compared to individuals who resolve HBV infection. Additionally, circulating IP-10 (3.72 vs. 1.12, p=0.015, median fold-change over baseline), IL-10 (7.03 vs. 1.0, p=0.003, median fold-change over baseline), and IL-18 (1.93 vs. 1.07, p=0.007, median fold-change over baseline) are elevated at visit 2 in HIV-uninfected individuals who develop persistent HBV infection.

To assess overall patterns of cytokine production associated with HBV persistence or recovery, heat maps of correlation coefficients were created to identify highly correlated groups of cytokine changes at visit 2 by HIV status and HBV outcome (Figure

4.5). Across all four groups, visit 2 fold-changes of three cytokines: IL-10, IP-10, and IL-18, are highly correlated. Further, TNF, MIP1α and MIP1β are highly correlated at visit 2 in both HIV-infected and HIV-uninfected individuals who develop persistent infection.

4.3.4 Principal Component Analysis identifies five immune factors

To overcome the complexities of building accurate multivariate models with highly collinear variables, principal component analysis (PCA) was employed as an unbiased approach to examine the influence of cytokine changes observed at visit 2 on observed variance in this dataset. Mean-centered visit 2 values (fold-change over baseline) for MIP1α, TNF, IP-10, MIP1β, IL-10, IL-18, eotaxin, IL-8, MCP4, MCP1, IFNγ, IL-15, IL-37, IL-7, and CCL17 were included in the PCA analysis. Five significant immune factors (eigen values>1) were included in the final analysis and collectively accounted for 70.8% of the variability within the dataset. The weighted loading values for each cytokine across the five immune factors are presented in Table 4.10.

Immune factor 1 accounted for 30.9% of the variance within the dataset and was heavily weighted by pro-inflammatory cytokines (including MIP1α and IP-10) and IL-10, which were associated at visit 2 with HBV persistence in the univariate analysis (Figure 4.6). Logistic regression of HBV outcome found that immune factors 1 (OR=2.63, 95%CI: 1.17-5.91) and 4 (OR: 2.15, 95% CI: 1.01-4.60) significantly predicted persistence after accounting for race, age, BMI, and HIV-status. When individuals were plotted by their unique values for factors 1, 2, and 3, which accounted for 56.5% of the total variance, there was a distinct cluster of individuals who had high values for high immune factor 1

amongst HIV-uninfected individuals that was not observed in HIV-infected individuals (Figure 4.7).

Because many pro-inflammatory cytokines contribute to immune factor 1, it was suspected that individuals with higher AST (AST >32U/L) might account for the clustering observed in HIV-uninfected individuals. While high AST perfectly predicted high immune factor 1 clustering in HIV-uninfected individuals who develop persistent HBV infection, high AST was not a predictor for high factor 1 clustering in HIV-uninfected individuals who recover from acute HBV (Figure 4.7).

4.3.5 Characterization of three unique stages of acute infection

In order to further characterize the acute immune response to HBV infection in this cohort, serological markers were used to divide the acute phase of infection into three distinct categories. Following the incubation period (~8 weeks), HBsAg is detectable in individuals with acute HBV for approximately 4 weeks prior to development of antibodies against core antigen (anti-HBc). Thus, early acute stage (EA) was defined serologically as HBsAg positive/ anti-HBc negative. The immune active acute stage of HBV infection (IAA) was defined serologically as positive for both HBsAg and anti-HBc; samples captured at this stage were estimated to be close to the time antibodies against HBcAg developed. A third category of acute infection was reserved for individuals who had no detectable HBsAg (SSC for suspected seroclearance) and was defined serologically as negative for HBsAg and anti-HBc positive. Individuals classified within the SSC category may be in later stages of acute self-limited infection, having progressed

through the EA and IA stages with subsequent seroclearance of HBsAg; alternatively, it is possible that they developed antibodies against HBcAg in the absence of detectable circulating HBsAg, a phenomenon observed in animal models where lower doses of viral inoculum contribute to robust T cell responses in the absence of circulating viral antigens[92].

The distribution of individuals by HIV status and HBV outcome in the three categories of acute infection is outlined in Table 4.11. Because individuals who develop persistent infection never seroclear HBsAg, there are no persisters in the SSC category of acute infection. There were no significant differences in AST values by HBV outcome in HIV-infected and HIV-uninfected individuals in the EA and IAA stage of acute infection, but circulating HBV DNA was significantly higher in HIV-uninfected individuals with persistent HBV as compared to HIV-uninfected individuals who recovered (7.9 vs. 4.4, median log10 copies/ml).

When individuals within the EA and IAA stage of acute infection at visit 2 were plotted by immune factors 1, 2, and 3, eleven HIV-uninfected individuals clustered high in immune factors 1 and 2 (Figure 4.8). Of these eleven, ten (91%) were captured in the IAA stage of infection and included both individuals who later recovered and those that developed persistent infection.

4.3.6 Circulating pro-inflammatory cytokines elevated in Immune Active Acute Infection

The changes in circulating cytokines at visit 2 (fold-change over the individual's baseline values) for HIV-uninfected individuals at each of the three stages of acute infection are reported in Table 4.12 Individuals in the immune active acute (IAA) stage of HBV infection had significant Visit 2 increases in circulating pro-inflammatory cytokines as compared to Visit 2 changes observed in individuals captured in the early acute (EA) and suspected seroconversion (SSC) stages of acute infection, including TNF (IAA: 1.73 vs. EA: 1.30 and SSC: 1.03, median fold-change, p=0.003), IL-10 (IAA:4.37 vs. EA:1.00 and SSC:1.00, median fold-change, p=0.014), IL-18 (IAA: 2.20 vs. EA: 1.11 and SSC: 1.06, median fold-change, p=0.007), and IP-10 (IAA: 5.23 vs. EA: 1.14 and SSC: 1.10, median fold-change, p=0.009). The chemokines MIP1α (EA:1.31 and IAA:2.03 vs. SSC:0.99, median fold-change, p=.002) and MIP1β (EA:1.39 and IAA:1.81 vs. SSC:0.96, median fold-change, p=0.02) were significantly increased in individuals in both the EA and IAA stages of acute infection.

A comparison of cytokine fold-changes, stratified by acute stage, is reported for HIV-uninfected individual by HBV outcome in Table 4.13. For individuals captured in the early acute (EA) and immune active (IAA) stages of acute infection, cytokine fold-changes observed at visit 2 were similar in HIV-uninfected individuals who recovered and those that developed persistent HBV infection. However, **for those captured during early acute (EA) infection,** CCL17 was significantly **elevated** in individuals **who develop** persistent HBV as compared to individuals who recover (1.25, IQR: 1.16-1.36,

vs. 0.88, IQR 0.75-1.08, fold-change over baseline, p=0.016, **Figure 4.9**). There was no significant difference in visit 2 fold-changes of CCL17 observed between individuals who recovered and those that developed persistent HBV infection captured in the IAA stage of infection. By visit 3, HIV-uninfected individuals with persistent HBV had significantly lower circulating CCL17 than HIV-infected individuals who recovered (Table 4.9A, 0.78,IQR:0.58-0.94, vs. 1.01, IQR: 0.81-1.29, fold-change over baseline, p=0.042).

4.3.7 HBV persistence associated with prolonged changes in circulating cytokines and chemokines

Changes in circulating cytokines were measured 6-20 months following estimated date of incident HBV infection at visit 3. Of note, fold-changes in IL-10 and IL-18 remain significantly elevated at visit 3 in individuals with persistent HBV infection (IL-10 fold-change over baseline: 8.47, IQR:2.77-13.5, vs. 1.0, IQR:1.0-1.0, p<0.001 in HIV-uninfected individuals and 1.83,IQR:1.11-5.50, vs. 1.00, IQR:0.75-1.69, p=0.003 in HIV-infected individuals and IL-18 fold change over baseline: 2.15, IQR:1.57-3.29, vs. 1.04, IQR:0.87-1.22, p<0.001 and 1.43, IQR:1.16-1.91, vs. 1.04, IQR:0.82-1.36, p=0.006 in HIV-uninfected and HIV-infected individuals, respectively, Table 4.9). IP-10 was no longer elevated in HIV-uninfected individuals who develop persistent HBV infection at visit 3, but MIP1α was still elevated at visit 3 in individuals who develop persistent infection as compared to those who recover from acute HBV infection (fold-change over baseline: 1.63, IQR: 1.15-1.98, vs. 1.01, IQR: 0.82-1.24, p=0.005 in HIV-uninfected individuals).

As mentioned previously, CCL17 was significantly decreased at visit 3 in HIV-uninfected individuals with persistent HBV as compared to individuals with HBV recovery, and also trended in this direction at visit 3 in HIV-infected individuals (visit 3 fold-change over baseline: 0.88, IQR: 0.66-1.08, vs. 1.14, IQR:0.90-1.42, p=0.09, Table 4.9). MCP-4 was also significantly decreased in HIV-infected individuals with persistent HBV, and trended lower in HIV-uninfected individuals with persistent HBV as well (visit 3 fold-change over baseline: 0.91, IQR:0.77-1.06, vs. 1.19, IQR:0.88-1.40, and 0.72, IQR:0.61-0.88, vs. 0.99, IQR:0.81-1.26, p=0.09, in HIV-infected and HIV-uninfected individuals, respectively).

4.4 Discussion

The extent to which peripheral cytokines and chemokines detected in symptomatic acute HBV infection mark immune function essential to control acute HBV infection, and which are unique to individuals who recover, has not been well characterized. Utilizing a cohort of 173 men with incident HBV infection, it was found that cytokine biomarkers in the earliest stages of acute HBV were strikingly similar in individuals who develop peristent infection and those who recover. The finding of elevated IP-10 and IL-10 at peak viremia is consistent with other studies of acute infection[228], but does not distinguish between recovery and persistent infection. Instead, increased peripheral CCL17 at the earliest stage of acute infection (HBsAg+/anti-HBc-) was unique to individuals who develop persistent infection. The chemokine receptor CCR4, to which CCL17 binds, is important for regulatory T cell recruitment into the liver[276], suggesting a potential mechanistic role for CCL17 in development of CHB.

To better understand the acute phase response to HBV infection given the highly collinear nature of cytokine data, this study employed principal component analysis and found five immune factors responsible for >70% of the variance within the dataset. The most significant of these, immune factor 1, was heavily weighted by acute-phase changes in several pro-inflammatory cytokines and chemokines, and IL-10, and was responsible for over 30% of the variance within the dataset. While a greater proportion (5 of 10, 50%) of the HIV-uninfected individuals who developed persistent HBV clustered into a group with high immune factor 1, 10% of HIV-uninfected individuals who recovered from acute infection also clustered into this group, suggesting this profile was not unique to HBV outcome. Indeed, stratification of acute HBV infection (visit 2) by early (HBsAg+/ anti-HBc-) and immune active (HBsAg+/anti-HBc+) stages established that cytokines associated with immune factor 1- namely IL-10, IL-18, IP-10, TNF, MIP1α, and MIP1β, were elevated in HIV-uninfected individuals in the immune active stage of acute infection and were not significantly different between individuals who recovered and those that developed CHB. These pro-inflammatory cytokines returned to baseline levels upon resolution of HBV infection in individuals who resolved acute infection, but IL-10, IL-18, and MIP1α remained elevated at visit 3 in those with persistent HBV infection.

The finding of elevated peripheral CCL17 during the earliest stage of acute infection (HBsAg+/ anti-HBc) in those who develop chronic infection is interesting in light of the role for this chemokine in recruitment of regulatory T cells (Tregs) to sites of inflammation. CCL17, also known as TARC, is produced primarily by myeloid-derived

dendritic cells (mDCs) and binds to its cognate receptor CCR4 expressed on Th2, Th17 and Tregs[277]. In a study using donor-matched blood and liver samples, Oo et al (2010) demonstrated that liver Tregs express significantly higher levels of the chemokine receptors CXCR3 and CCR4 than peripheral Tregs. Further, they demonstrated that CCL17 was detectable exclusively in inflamed livers and was derived from dendritic cells co-localizing with CD8+ T cells and Tregs present in inflammatory infiltrates, suggesting that CCL17 facilitates co-localization of regulatory T cells with Teff cells in the inflamed liver.[276]

While little is known about the role of CCL17 in HBV infection, CCL17 expression is increased in the liver of patients with chronic hepatitis C, and correlates with increased regulatory T cells in liver biopsies[278]. Similar to findings in chronic hepatitis C, regulatory T cell numbers are significantly higher in liver biopsies from individuals with CHB as compared to healthy controls.[279] Peripheral Tregs are increased in CHB, correlate with HBV DNA, and have been shown to decrease significantly in individuals with viral suppression following entecavir or adefovir treatment.[279–281]

In an murine model of acute HBV using AdHBV, early depletion of regulatory T cells (immediately prior to infection and at Day 2 following infection, but not at one week following infection) resulted in enhanced cytokine production by HBV-specific CD8+T cells, significantly lower peripheral HBV DNA by day 7, and earlier seroclearance of HBeAg and HBsAg.[139] This model suggests there may be a critical window for intrahepatic Treg accumulation in the liver in control of acute HBV. As much of the adaptive immune response to HBV infection is primed in the liver microenvironment[282],

it is plausible that early accumulation of regulatory T cells in the liver would lead to development of poor T cell responses and impaired antibody production and class-switching in HBV infection. Somewhat paradoxically, antibody-mediated blockade of CCR4 as part of a chemotherapeutic regimen for adult T-cell leukemia/ lymphoma has resulted in several reports of recrudescent HBV infection, suggesting a multi-faceted role for regulatory T cells dependent on the stage of HBV control.[283–285]

Animal models, including the HBV-transgenic mouse and our own findings in the murine AdHBV model of acute infection (presented in Chapter 3), have shown that increases in liver inflammation and serum liver enzymes are associated with a secondary liver-infiltration of pro-inflammatory macrophages rather than increased intrahepatic CD8+T cells[286]. In this study, serum AST was not significantly elevated in individuals with persistent infection until the later stage of infection (visit 3), consistent with a model of persistent HBV replication and gene expression in the absence of functional anti-HBV adaptive responses.

While this study identified some overlap in the acute immune response to HBV infection in HIV-uninfected and HIV-infected individuals, it also found certain parameters were significantly altered in HIV-infected individuals- ostensibly due to immune suppression and persistent HIV viral replication. It is known that HIV-coinfection increases the likelihood of persistent HBV infection and worsens outcomes associated with CHB[287], but it is outside the scope of this thesis to fully dissect the role HIV-coinfection plays in modulating the acute immune response to HBV infection. It is of note that certain parameters correlated with higher HIV RNA (e.g. CCL17) and others with CD4+numbers

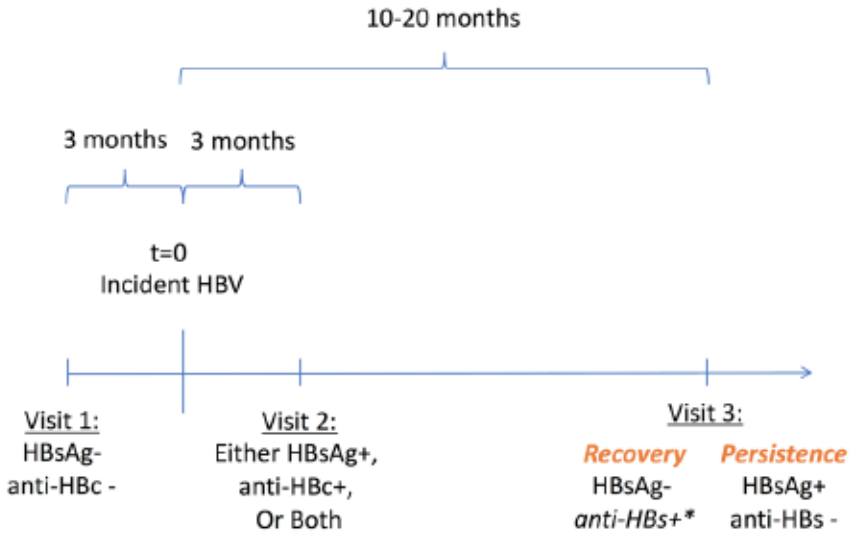
(e.g. IL-10), and that these correlations are not unexpected given the cellular sources of these cytokines and chemokines. Still, a more robust statistical analysis that includes additional parameters (e.g. anti-retroviral therapy, ART) will be required to elucidate potential mechanisms of control in HIV-infected individuals, and will be pursued as part of future directions.

It should be noted that the findings of this study are limited to the study of peripheral cytokines and chemokines, and may hint at but not correlate with immune function in the liver. In this regard, peripheral chemokine data is generally more reliable than cytokine data, as cytokines act locally in an autocrine or paracrine fashion; thus systemic detection may be a better indicator of liver tissue damage, which results in leakage of these cytokines from the local microenvironment into the periphery. Interpretation of these results is further complicated by the transient nature of cytokine/ chemokine expression, the presence of soluble decoy receptors (e.g. IL2r) that inactivate excess cytokine, and by differential activity of cytokines dependent on presentation and the redundancy in function between certain cytokines and chemokines.[180,288–290]

This study is unique in that it captures individuals who develop CHB in the acute stage of incident infection, and demonstrates that much of the immune response that has been documented in previous cohorts of symptomatic acute infection is not unique to acute self-limited hepatitis B. This study builds upon other research by characterizing the immune response at different stages of acute infection. It further identifies elevated peripheral CCL17 in early stages of acute infection as an immune response unique to

individuals who develop chronic infection, and suggests more study on the contribution of CCL17 to intrahepatic Treg accumulation in the context of HBV infection is warranted.

Figure 4.1: Study Design- Sampling and Serological Outcomes by Visit



*anti-HBs antibodies may or may not be present at detectable levels in people with Acute Self-limited HBV infection

Figure 4.2: Time from estimated incident HBV infection for visits 1, 2, and 3 by HIV status and HBV outcome

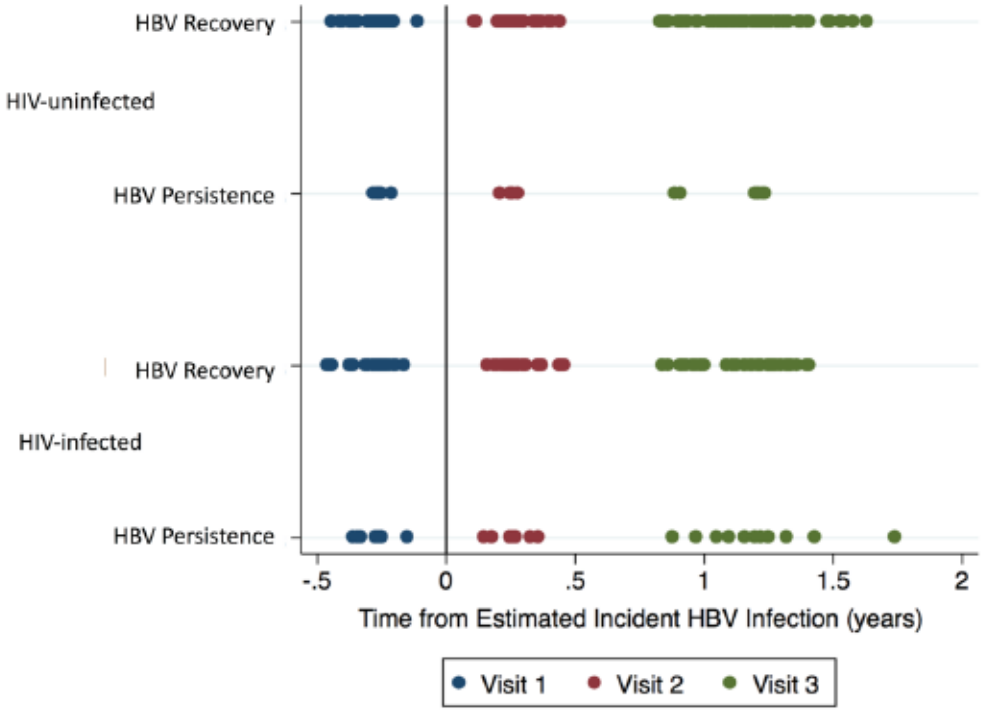


Table 4.1: Sample availability by HIV status and HBV outcome

		Visits 1, 2, & 3	Visits 1 & 2 only	Visits 2 & 3 only
HIV-uninfected	Controller (n=100)	91 (91.0%)	8 (8.0%)	1 (1.0%)
	Non-Controller (n=10)	8 (80.0%)	2 (20.0%)	0 (0.0%)
HIV-infected	Controller (n=51)	48 (94.1%)	1 (2.0%)	2 (3.9%)
	Non-Controller (n=12)	12 (100.0%)	0 (0.0%)	0 (0.0%)

Table 4. 2: Summary of Assay Type by Analyte

Analyte	Assay Type	Manufacturer	LLOQ (pg/ml)
IFNγ	**V-plex pro-inflammatory**	**MSD**	**5.4**
IL-1β	V-plex pro-inflammatory	MSD	2.14
IL-2	V-plex pro-inflammatory	MSD	0.90
IL-4	V-plex pro-inflammatory	MSD	0.45
IL-6	V-plex pro-inflammatory	MSD	0.72
IL-8	**V-plex pro-inflammatory**	**MSD**	**1.13**
IL-10	**V-plex pro-inflammatory**	**MSD**	**0.3**
IL-12	V-plex pro-inflammatory	MSD	0.4
IL-13	V-plex pro-inflammatory	MSD	2.5
TNF	**V-plex pro-inflammatory**	**MSD**	**0.5**
IL-5	V-plex Cytokines	MSD	2.0
IL-7	**V-plex Cytokines**	**MSD**	**1.37**
IL-15	V-plex Cytokines	MSD	0.7
LTα	V-plex Cytokines	MSD	0.5
Eotaxin (CCL11)	**V-plex Chemokines**	**MSD**	**12.3**
IP-10 (CXCL10)	**V-plex Chemokines**	**MSD**	**1.37**
Mip1α (CCL3)	**V-plex Chemokines**	**MSD**	**13.8**
Mip1β (CCL4)	**V-plex Chemokines**	**MSD**	**2.27**
MCP1 (CCL2)	**V-plex Chemokines**	**MSD**	**1.09**
MCP4 (CCL13)	**V-plex Chemokines**	**MSD**	**5.13**
TARC (CCL17)	**V-plex Chemokines**	**MSD**	**3.32**
IFNα	U-plex w/ IL18	MSD	10
IL-18	**U-plex w/ IFNa**	**MSD**	**10**
IL-37	**ELISA**	**Adipogen**	**200**

Table 4.3: Number of samples detected above LLOQ for each analyte by visit, (% detectable)

Grey shading indicates <30% of samples were above the LLOQ, and these analytes were not further analzed.

	Visit 1 (n=171)	Visit 2 (n=175)	Visit 3 (n=164)	Total (n=510)	½ of LLOQ (pg/ml)
IFNα	0 (0.0%)	2 (1.1%)	1 (0.6%)	3 (0.6%)	5.0
IFNγ	59 (34.5%)	66 (37.7%)	56 (34.2%)	181 (35.5%)	2.7
IL-1β	3 (1.8%)	2 (1.1%)	1 (0.6%)	6 (1.2%)	1.07
IL-2	7 (4.1%)	7 4.0%)	5 (3.1%)	19 3.4%)	0.45
IL-4	0 (0.0%)	1 (0.6%)	2 (1.2%)	3 (0.6%)	0.225
IL-5	4 (2.5%)	3 (1.7%)	1 (0.6%)	8 (1.6%)	1.0
IL-6	47 (28.9%)	37 (21.6%)	44 (25.4%)	128 (25.1%)	0.36
IL-7	171 (100.0%)	175 (100.0%)	164 (100.0%)	510 (100.0%)	n/a
IL-8	171 (100.0%)	174 (99.4%)	164 (100.0%)	509 (99.7%)	0.565
IL-10	76 (44.4%)	109 (62.3%)	86 (52.4%)	271 53.1%)	0.15
IL-12	18 (10.5%)	17 (9.7%)	11 (6.7%)	46 (9.0%)	0.20
IL-13	8 (4.7%)	7 (4.0%)	6 (3.7%)	21 (4.1%)	1.25
IL-15	171 (100.0%)	175 (100.0%)	164 (100.0%)	510 (100.0%)	n/a
IL-18	171 (100.0%)	175 (100.0%)	164 (100.0%)	510 (100.0%)	5.0
IL-37	98 (57.3%)	98 (56.0%)	88 (53.7%)	284 (55.7%)	100
TNF	170 (99.4%)	174 (99.4%)	163 (99.4%)	507 (99.4%)	0.25
LTα	1 (0.6%)	4 (2.3%)	3 (1.8%)	8 (1.6%)	0.25
Eotaxin (CCL11)	171 (100.0%)	175 (100.0%)	164 (100.0%)	510 (100.0%)	n/a
IP-10 (CXCL10)	171 (100.0%)	175 (100.0%)	164 (100.0%)	510 (100.0%)	n/a
Mip1α (CCL3)	171 (100.0%)	175 (100.0%)	164 (100.0%)	510 (100.0%)	n/a
Mip1β (CCL4)	163 (95.3%)	170 (97.1%)	152 (92.7%)	489 (95.1%)	1.135
MCP1 (CCL2)	171 (100.0%)	175 (100.0%)	164 (100.0%)	510 (100.0%)	n/a
MCP4 (CCL13)	171 (100.0%)	175 (100.0%)	164 (100.0%)	510 (100.0%)	n/a
TARC (CCL17)	171 (100.0%)	175 (100.0%)	164 (100.0%)	510 (100.0%)	n/a

Table 4.4: Characteristics of Cohort at Baseline (Visit 1)

Values are presented as Median (Interquartile Range), or N(%). P Values from univariate Mann-Whitney or Kruskall-Wallis tests (race), stratified by HIV status, comparing individuals who later experience HBV recovery versus those that develop persistent infection following incident HBV infection.

	HIV Uninfected			HIV Infected		
	HBV Recovery (n=100)	HBV Persistence (n=10)	P	HBV Recovery (n=50)	HBV Persistence (n=12)	P
Age (years)	31 (27-39)	32 (24-36)	0.5	37 (31-44)	33 (28-43)	0.4
Race						
White	91 (91.0%)	10 (100.0%)	0.4	37 (74.0%)	32 (91.7%)	0.2
Black	4 (4.0%)	0 (0.0%)		9 (18.0%)	1 (8.3%)	
Other	5 (5.0%)	0 (0.0%)		4 (8.0%)	0 (0.0%)	
AST	18.6 (14.2-25.5)	15.9 (12.1-18.6)	0.2	21.9 (16.3-27.1)	22.8 (17.0-30.3)	0.7
BMI	24 (21.5-26)	24 (22-25)	1.0	24 (21-26)	23 (22-24.5)	0.4

Table 4.5: Baseline (Visit 1) Cytokine and Chemokine Concentrations by HIV status and HBV outcome.
Values represent Median (Interquartile Range). P Values from univariate rank-sum tests, stratified by HIV status, comparing Individuals who later experience HBV recovery versus those that develop persistent infection following incident HBV infection.

Analyte	Detectable N (%)	HIV Uninfected HBV Recovery (n=99)	HIV Uninfected HBV Persistence (n=10)	p- value	HIV Infected HBV Recovery (n=50)	HIV Infected HBV Persistence (n=12)	p- value
IFNγ	59 (35%)	2.7 (2.7-2.7)	2.7 (2.7-6.4)	0.198	6.1 (2.7-9.3)	2.7 (2.7-7.5)	0.227
IL-7	171 (100%)	15.9 (11.3-20.9)	12.6 (9.3-13.6)	0.123	17.0 (13.2-22.8)	16.0 (11.7-23.6)	0.587
IL-8	171 (100%)	14.1 (9.1-24.6)	8.8 (8.2-21.0)	0.240	14.1 (9.8-24.7)	9.3 (7.9-11.7)	0.018
IL-10	76 (44%)	0.2 (0.2-0.4)	0.2 (0.2-0.4)	0.552	0.4 (0.2-0.6)	0.4 (0.2-0.6)	0.520
IL-15	171 (100%)	1.9 (1.7-2.4)	2.4 (2.1-2.7)	0.032	2.2 (1.9-2.6)	2.0 (1.7-2.5)	0.454
IL-18	171 (100%)	432.9 (315.4-576.8)	387.6 (267.6-559.2)	0.522	691.4 (506.9-1204.5)	687.9 (524.3-1089.0)	0.744
IL-37	98 (57%)	407.0 (100.0-995.0)	243.6 (100.0-777.6)	0.344	252.4 (100.0-687.2)	176.0 (100.0-1005.8)	0.909
TNF	170 (99%)	1.6 (1.2-2.0)	1.4 (1.1-1.8)	0.342	2.74 (1.89-3.87)	2.27 (1.68-3.64)	0.551
IP10	171 (100%)	149.0 (91.9-178.4)	166.1 (143.8-182.9)	0.280	324.0 (225.8-599.4)	448.3 (215.5-605.3)	0.708
Eotaxin	171 (100%)	133.1 (104.5-180.2)	117.7 (73.4-149.2)	0.193	161.6 (113.6-253.8)	134.6 (104.2-235.3)	0.581
MCP-1	171 (100%)	291.7 (232.7-355.7)	298.8 (237.2-366.7)	0.652	290.3 (229.8-389.6)	338.6 (284.9-387.0)	0.498
MCP-4	171 (100%)	70.2 (53.8-100.1)	82.8 (75.0-94.2)	0.505	63.4 (47.3-110.9)	84.9 (58.2-114.0)	0.159
MIP1α	171 (100%)	103.0 (75.0-134.0)	87.5 (80.2-153.1)	0.767	88.5 (69.6-144.7)	82.9 (79.2-105.3)	0.762
MIP1β	163 (95%)	14.7 (9.3-20.4)	14.4 (12.1-23.1)	0.686	19.1 (12.0-28.8)	16.5 (11.4-18.8)	0.323
CCL17	171 (100%)	273.2 (185.4-447.9)	274.1 (202.6-445.4)	0.933	273.6 (166.2-325.7)	188.0 (145.0-372.2)	0.817

Table 4.6: Serum AST at Visits 1, 2, and 3

Values represent Median (Interquartile Range). P Values from univariate rank-sum tests, stratified by HIV status, comparing individuals who later experience HBV recovery versus those that develop persistent infection following incident HBV infection

	HIV Uninfected			HIV Infected		
	HBV Recovery (n=101)	HBV Persistence (n=10)	p	HBV Recovery (n=51)	HBV Persistence (n=12)	p
v1	18.6 (14.2-25.7)	15.0 (12.1-18.6)	0.11	21.9 (16.3-27.1)	22.8 (17.0-30.3)	0.70
v2	18.1 (13.6-29.1)	25.9 (12.4-158.8)	0.27	22.5 (16.1-28.6)	25.7 (13.7-55.9)	0.52
v3	19.3 (14.4-24.8)	52.3 (27.9-187.3)	0.008	22.4 (18.1-37.9)	46.9 (30.6-93.6)	0.002

Figure 4.3: Serum AST trends following acute hepatitis B infection.
(A) Serum AST by HIV-status and HBV outcome (box plot shows Median & IQR, whiskers show min/max values within 1.5xIQR, outliers shown as individual dots), (B) Visit 2 AST plotted against HBV DNA (log10 copies/ml), each dot indicates a single person.

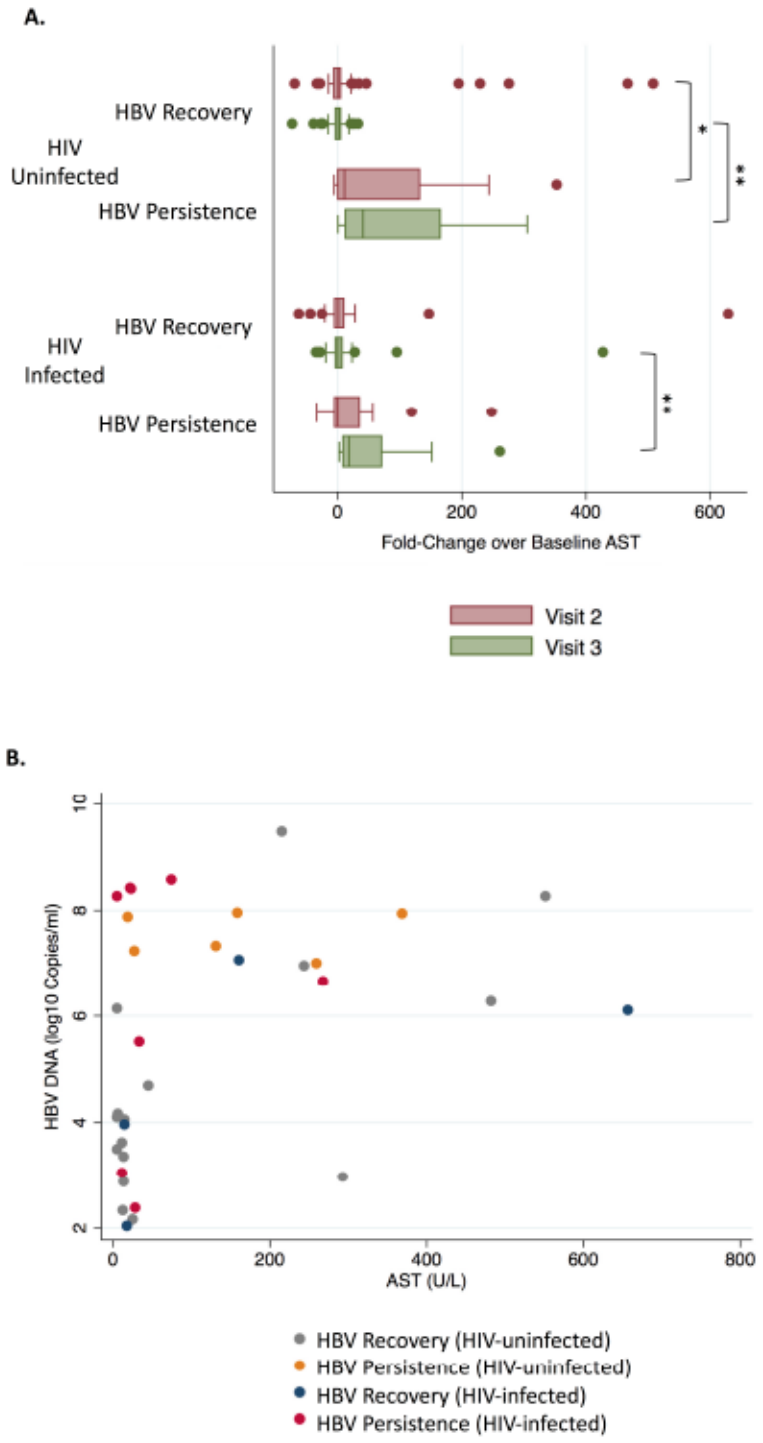


Table 4.7: Cytokine/ Chemokine Values at Visit 2

Values represent Median (Interquartile Range) pg/ml. P Values from univariate rank-sum tests, stratified by HIV status, comparing individuals who later experience HBV recovery versus those that develop persistent infection following incident HBV infection

Analyte	HIV Uninfected			HIV Infected		
	HBV Resolved (n=100)	HBV Persistent (n=10)	p- value	HBV Resolved (n=50)	HBV Persistent (n=12)	p- value
IFNγ	2.7 (2.7-2.7)	2.7 (2.7-5.8)	0.865	6.6 (2.7-9.5)	8.0 (2.7-10.1)	0.610
IL-7	15.1 (11.2-20.8)	14.3 (11.0-19.9)	0.827	16.5 (13.4-23.1)	16.3 (11.4-22.0)	0.700
IL-8	13.6 (8.5-26.5)	14.2 (11.6-85.2)	0.493	11.9 (8.5-19.7)	11.2 (8.8-16.3)	0.569
IL-10	**0.3 (0.2-0.6)**	**2.2 (0.3-4.4)**	**0.006**	0.5 (0.2-0.7)	0.9 (0.4-1.1)	0.052
IL-15	2.0 (1.7-2.5)	2.3 (2.3-2.3-2.7)	0.049	2.2 (1.9-2.6)	2.1 (2.0-2.6)	0.958
IL-18	494.0 (373.6-607.6)	835.7 (399.4-1575.6)	0.146	658.5 (534.3-1293.1)	888.6 (625.1-1186.4)	0.552
IL-37	535.2 (100.0-1170.0)	446.8 (100.0-608.7)	0.455	100.0 (100.0-843.2)	221.4 (100.0-996.1)	0.729
TNF	1.9 (1.2-2.4)	2.0 (1.8-2.3)	0.529	2.8 (1.8-4.3)	3.5 (2.1-5.2)	0.244
IP10	**156.0 (108.6-240.7)**	**580.9 (192.2-882.7)**	**0.010**	346.7 (178.1-627.5)	411.3 (280.0-1169.4)	0.183
Eotaxin	141.4 (111.6-188.7)	128.3 (88.1-179.1)	0.262	152.5 (112.4-230.7)	149.6 (110.1-251.9)	0.903
MCP-1	302.0 (231.5-364.1)	372.4 (300.2-426.8)	0.039	307.4 (236.2-396.2)	383.8 (279.1-423.3)	0.363
MCP-4	66.9 (54.2-92.6)	68.6 (51.4-90.0)	0.893	65.9 (47.5-92.3)	67.0 (55.4-108.4)	0.558
MIP1α	**106.2 (78.5-155.0)**	**223.7 (121.7-268.1)**	**0.002**	**86.2 (69.6-125.2)**	**124.6 (87.6-167.0)**	**0.077**
MIP1β	**15.3 (8.6-25.9)**	**27.1 (19.0-31.6)**	**0.022**	19.2 (14.0-27.7)	19.6 (16.4-40.8)	0.336
CCL17	275.0 (185.6-447.2)	318.0 (156.0-398.2)	0.892	243.5 (149.9-362.5)	219.1 (166.2-316.3)	0.624

Table 4.8: Cytokine/ Chemokine Values at Visit 3

Values represent Median (Interquartile Range) pg/ml. P Values from univariate rank-sum tests, stratified by HIV status, comparing individuals who later experience HBV recovery versus those that develop persistent infection following incident HBV infection

Analyte	HIV Uninfected			HIV Infected		
	HBV Recovery (n=93)	HBV Persistence (n=8)	p- value	HBV Recovery (n=50)	HBV Persistence (n=12)	p- value
IFNγ	2.7 (2.7-2.7)	2.7 (2.7-4.2)	0.632	5.5 (2.7-9.5)	6.8 (5.8-9.3)	0.253
IL-7	15.8 (11.5-19.2)	13.2 (7.4-13.7)	0.068	17.9 (14.3-24.2)	18.2 (10.4-20.8)	0.289
IL-8	14.1 (8.1-26.3)	12.2 (7.8-18.2)	0.538	15.4 (10.6-20.7)	17.8 (10.2-22.7)	0.593
IL-10	**0.2 (0.2-0.2)**	**1.7 (0.4-2.5)**	**<0.001**	**1.13 (0.69-1.48)**	**1.08 (1.11-4.78)**	**0.008**
IL-15	2.0 (1.7-2.5)	2.2 (2.2-2.9)	0.079	2.2 (1.8-2.5)	2.4 (2.0-2.7)	0.273
IL-18	**449.7 (314.0-566.9)**	**677.6 (448.6-1423.6)**	**0.011**	704.6 (567.7-1095.4)	1036.9 (740.6-1439.2)	0.094
IL-37	333.0 (100.0-946.4)	370.3 (195.4-573.2)	0.876	100.0 (100.0-691.6)	293.6 (100.0-801.0)	0.396
TNF	1.6 (1.2-2.0)	1.6 (1.5-2.1)	0.443	2.8 (1.9-3.7)	3.4 (2.7-5.0)	0.081
IP10	**138.8 (94.0-173.6)**	**226.4 (159.5-344.0)**	**0.012**	392.7 (235.7-660.2)	510.7 (493.5-862.1)	0.101
Eotaxin	134.0 (104.7-183.1)	106.2 (74.8-136.4)	0.163	171.5 (137.0-217.3)	182.3 (162.6-208.1)	0.742
MCP-1	305.9 (242.3-373.2)	304.5 (274.1-344.8)	0.801	326.3 (262.7-417.2)	363.7 (289.7-436.7)	0.397
MCP-4	69.3 (49.3-94.0)	59.2 (47.3-75.6)	0.400	84.0 (61.8-110.2)	66.0 (61.0-91.9)	0.277
MIP1α	**95.1 (72.2-139.4)**	**172.6 (119.7-262.8)**	**0.001**	88.1 (71.0-139.0)	123.9 (103.7-150.8)	0.069
MIP1β	14.6 (9.9-21.9)	21.3 (12.5-25.3)	0.191	20.1 (13.8-30.6)	24.0 (18.5-27.2)	0.359
CCL17	293.9 (189.7-455.3)	281.1 (179.4-353.5)	0.474	253.7 (172.7-389.8)	217.1 (138.6- 287.0)	0.091

Tables 4.9 A &B: Visit 2 and 3 Fold-Changes in Circulating Cytokines and Chemokines:
Plasma or serum cytokine/ chemokines levels measured pre-infection (visit 1), in early acute HBV infection (visit 2), and late acute infection/ recovery (visit 3). Values from visits 2 and 3 were divided by same-person pre-infection values (visit 1) to give fold-changes in circulating cytokines at visits 2 and 3. Data presented as Median (Interquartile Range).

Table 4.9 A: HIV Uninfected Individuals

Analyte		HBV Recovery*	HBV Persistence **	p [A]	p [B]
IFNγ	v2	1.0 (1.0-1.0)	1.0 (0.49-1.0)	0.259	0.265
	v3	1.0 (1.0-1.0)	1.0 (0.49-1.0)	0.547	0.976
IL-7	v2	**0.98 (0.78-1.18)**	**1.28 (1.01-1.45)**	**0.038**	0.143
	v3	0.93 (0.78-1.13)	1.01 (0.63-1.24)	0.889	0.836
IL-8	v2	*0.96 (0.56-1.70)*	*1.51 (1.09-2.30)*	*0.051*	*0.142*
	v3	0.96 (0.50-1.65)	1.28 (0.68-2.40)	0.517	0.645
IL-10	v2	**1.0 (1.0-2.40)**	**7.03 (2.13-17.80)**	**0.005**	**0.003**
	v3	**1.0 (1.0-1.0)**	**8.47 (2.77-13.50)**	**<0.001**	**<0.001**
IL-15	v2	0.99 (0.93-1.14)	0.97 (0.85-1.11)	0.592	0.834
	v3	1.03 (0.91-1.13)	0.95 (0.92-1.04)	0.446	0.694
IL-18	v2	**1.07 (0.91-1.31)**	**1.93 (1.11-3.04)**	**0.012**	**0.007**
	v3	**1.04 (0.87-1.22)**	**2.15 (1.57-3.29)**	**<0.001**	**0.007**
IL-37	v2	1.00 (0.96-1.02)	1.06 (1.00-1.49)	0.165	0.149
	v3	1.00 (0.82-1.00)	1.06 (0.95-2.97)	0.093	**0.018**
TNF	v2	1.07 (0.90-1.69)	1.5 (1.13-1.82)	0.108	0.306
	v3	0.98 (0.80-1.30)	1.26 (0.99-1.78)	0.077	0.117
IP10	v2	**1.12 (0.87-1.49)**	**3.72 (1.06-7.96)**	**0.063**	**0.015**
	v3	1.02 (0.80-1.34)	1.24 (0.99-2.60)	0.109	0.170
Eotaxin	v2	1.03 (0.82-1.31)	1.16 (0.75-1.67)	0.652	0.803
	v3	1.02 (0.80-1.34)	1.24 (0.99-2.61)	0.109	0.269
MCP-1	v2	*1.04 (0.90-1.18)*	*1.15 (1.01-1.46)*	*0.060*	0.085
	v3	1.00 (0.88-1.19)	1.21 (0.89-1.41)	0.347	0.395
MCP-4	v2	0.99 (0.81-1.17)	0.91 (0.65-1.03)	0.309	0.449
	v3	**0.99 (0.81-1.26)**	**0.72 (0.61-0.88)**	**0.019**	**0.092**
MIP1α	v2	**1.08 (0.83-1.35)**	**2.16 (1.34-3.20)**	**0.005**	***0.003***
	v3	**1.01 (0.82-1.24)**	**1.63 (1.15-1.98)**	**0.007**	**0.005**
MIP1β	v2	1.00 (0.72-1.91)	1.54 (0.97-3.12)	0.118	*0.086*
	v3	1.00 (0.73-1.58)	1.52 (0.77-1.95)	0.374	*0.238*
CCL17	v2	0.97 (0.84-1.15)	1.11 (0.74-1.25)	0.571	0.821
	v3	**1.01 (0.81-1.29)**	**0.78 (0.58-0.94)**	**0.028**	**0.042**

***N=99 at visit 2, 92 at visit 3**
****N=10 at visit 2, 8 at visit 3**

A. Unadjusted, Rank Sum Test
B. Logistic regression of log10(Visit2/Visit1) or log10(Visit2/Visit1), adjusting for BMI, race, and age

Table 4.9 B: HIV Infected Individuals

Analyte		HBV Recovery*	HBV Persistence **	p [A]	p [B]	p [C]	p [D]
IFNγ	**v2**	1.0 (0.82-1.34)	1.20 (1.1-2.34)	0.190	0.095	0.120	0.226
	v3	**1.0 (0.78-1.63)**	**1.33 (1.06-2.66)**	**0.023**	**0.060**	**0.057**	**0.035**
IL-7	**v2**	0.99 (0.83-1.10)	1.00 (0.77-1.25)	0.643	0.859	0.985	0.115
	v3	1.03 (0.93-1.21)	0.86 (0.78-1.12)	0.142	0.250	0.273	0.626
IL-8	**v2**	*1.06 (0.60-1.30)*	*1.22 (0.98-1.69)*	*0.069*	*0.038*	*0.074*	*0.047*
	v3	1.09 (0.68-1.58)	1.94 (1.28-2.13)	0.034	0.189	0.187	0.207
IL-10	**v2**	**1.13 (0.69-1.48)**	**1.08 (1.11-4.78)**	**0.043**	**0.032**	**0.028**	**0.033**
	v3	**1.00 (0.75-1.69)**	**1.83 (1.11-5.50)**	**0.003**	**0.004**	**0.006**	**0.013**
IL-15	**v2**	1.01 (0.88-1.14)	1.10 (1.03-1.21)	0.139	0.184	0.185	0.220
	v3	**1.00 (0.87-1.12)**	**1.10 (1.02-1.24)**	**0.062**	**0.037**	**0.030**	**0.056**
IL-18	**v2**	*1.03 (0.91-1.18)*	*1.14 (1.03-1.56)*	*0.078*	*0.076*	*0.059*	*0.052*
	v3	**1.04 (0.82-1.36)**	**1.43 (1.16-1.91)**	**0.006**	**0.009**	**0.006**	**0.019**
IL-37	**v2**	1.00 (0.92-1.00)	1.00 (0.87-1.00)	0.441	0.948	0.765	0.932
	v3	1.00 (0.83-1.00)	1.00 (0.75-1.00)	0.704	0.560	0.527	0.503
TNF	**v2**	**1.12 (0.81-1.23)**	**1.23 (1.11-2.25)**	**0.023**	**0.006**	**0.012**	**0.020**
	v3	**1.08 (0.80-1.28)**	**1.42 (1.30-1.71)**	**0.001**	**0.106**	**0.115**	**0.136**
IP10	**v2**	1.06 (0.60-1.48)	1.22 (0.90-1.73)	0.301	0.397	0.463	0.480
	v3	1.24 (0.70-1.83)	1.40 (1.07-2.17)	0.355	0.313	0.368	0.663
Eotaxin	**v2**	0.93 (0.79-1.15)	1.11 (0.87-1.51)	0.285	0.522	0.465	0.115
	v3	1.01 (0.84-1.35)	1.12 (0.78-1.70)	0.677	0.893	0.554	0.889
MCP-1	**v2**	1.01 (0.89-1.16)	1.04 (0.90-1.22)	0.643	0.736	0.946	0.658
	v3	1.06 (0.92-1.24)	1.11 (1.03-1.34)	0.346	0.299	0.208	0.133
MCP-4	**v2**	0.93 (0.78-1.21)	0.90 (0.67-1.13)	0.569	0.351	0.577	0.505
	v3	**1.19 (0.88-1.40)**	**0.91 (0.77-1.06)**	**0.023**	**0.039**	**0.076**	**0.059**
MIP1α	**v2**	**1.03 (0.82-1.20)**	**1.14 (1.01-2.02)**	**0.052**	**0.005**	**0.009**	**0.02**
	v3	**1.05 (0.78-1.28)**	**1.38 (1.17-1.69)**	**0.006**	**0.203**	**0.233**	**0.207**
MIP1β	**v2**	**1.00 (0.72-1.33)**	**1.34 (0.99-1.96)**	**0.067**	**0.018**	**0.035**	**0.046**
	v3	*0.98 (0.71-1.59)*	*1.62 (1.18-1.97)*	*0.092*	*0.459*	*0.400*	*0.573*
CCL17	**v2**	1.02 (0.74-1.24)	0.93 (0.80-1.15)	0.817	0.859	0.888	0.511
	v3	**1.14 (0.90-1.42)**	**0.88 (0.69-1.08)**	**0.037**	**0.088**	**0.115**	**0.324**

***N=50 at visit 2, 49 at visit 3**

****N=12 at visits 2 and 3**

A. Unadjusted, Rank Sum Test
B. Logistic regression of log10(Visit2/Visit1) or log10(Visit2/Visit1), adjusting for BMI, race, and age
C. Logistic regression as in b, adjusting for BMI, race, age, and CD4 count
D. Logistic regression as in b, adjusting for BMI, race, age, CD4 count, and HIV RNA

Figure 4.4: Visit 2 Cytokine Fold-changes over baseline.
Intra-person Fold-changes in Circulating Cytokines (A) and Chemokines (B) by HIV status and HBV outcome. Center line represents median, box represents inter-quartile range, whiskers represent min/ max samples within 1.5xIQR of the nearest quartile, individual dots represent outliers beyond 1.5xIQR from the nearest quartile, *=p-value<0.05 from Rank-Sum Test.

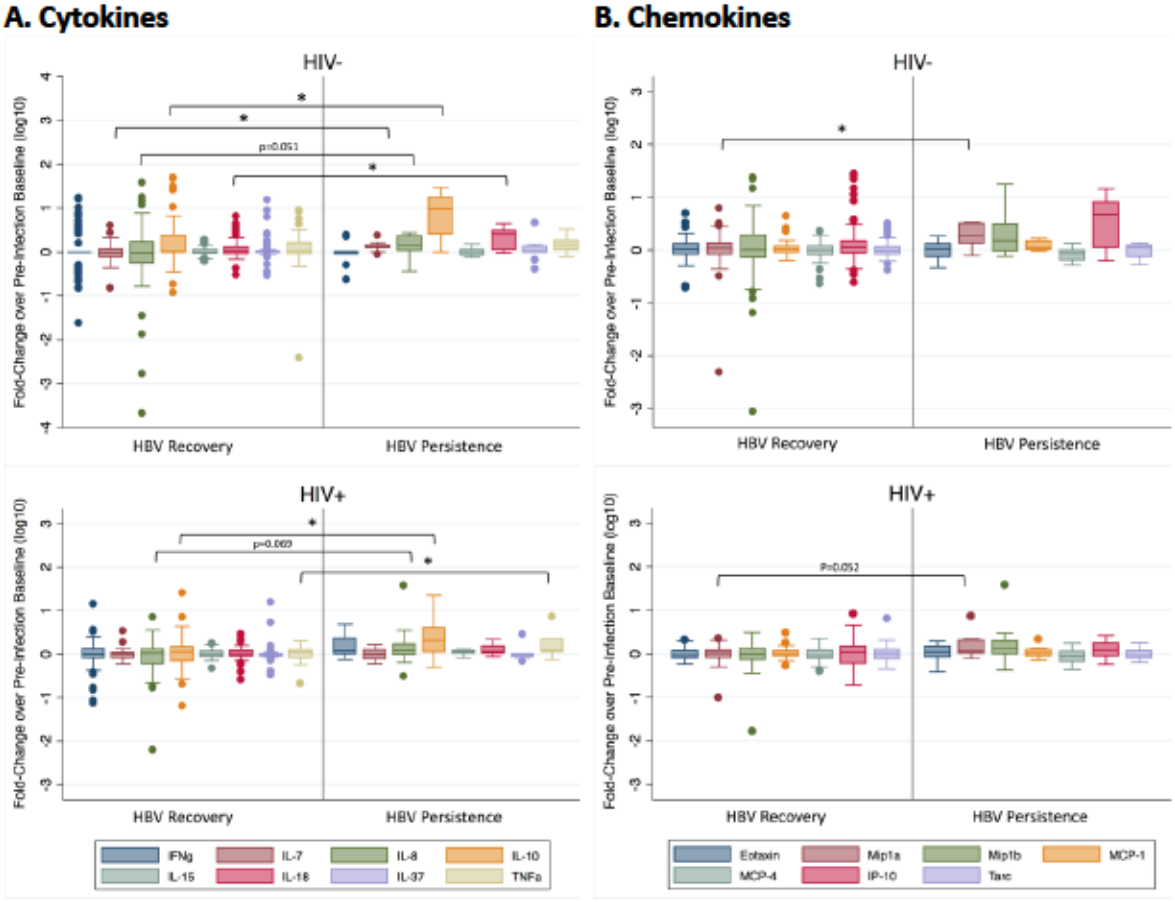


Figure 4.5: Heat-map of Correlations between Visit 2 Fold-change values for Cytokines and Chemokines by HIV-status and HBV outcome.

(A) HIV-uninfected w/ HBV recovery (n=99), (B) HIV-uninfected individuals w/ HBV persistence (n=10), (C) HIV-infected individuals w/ HBV recovery(n=50), and (D) HIV-infected individuals w/ HBV persistence (n=12). Green indicates a positive correlation, and red indicates a negative correlation between two cytokines.

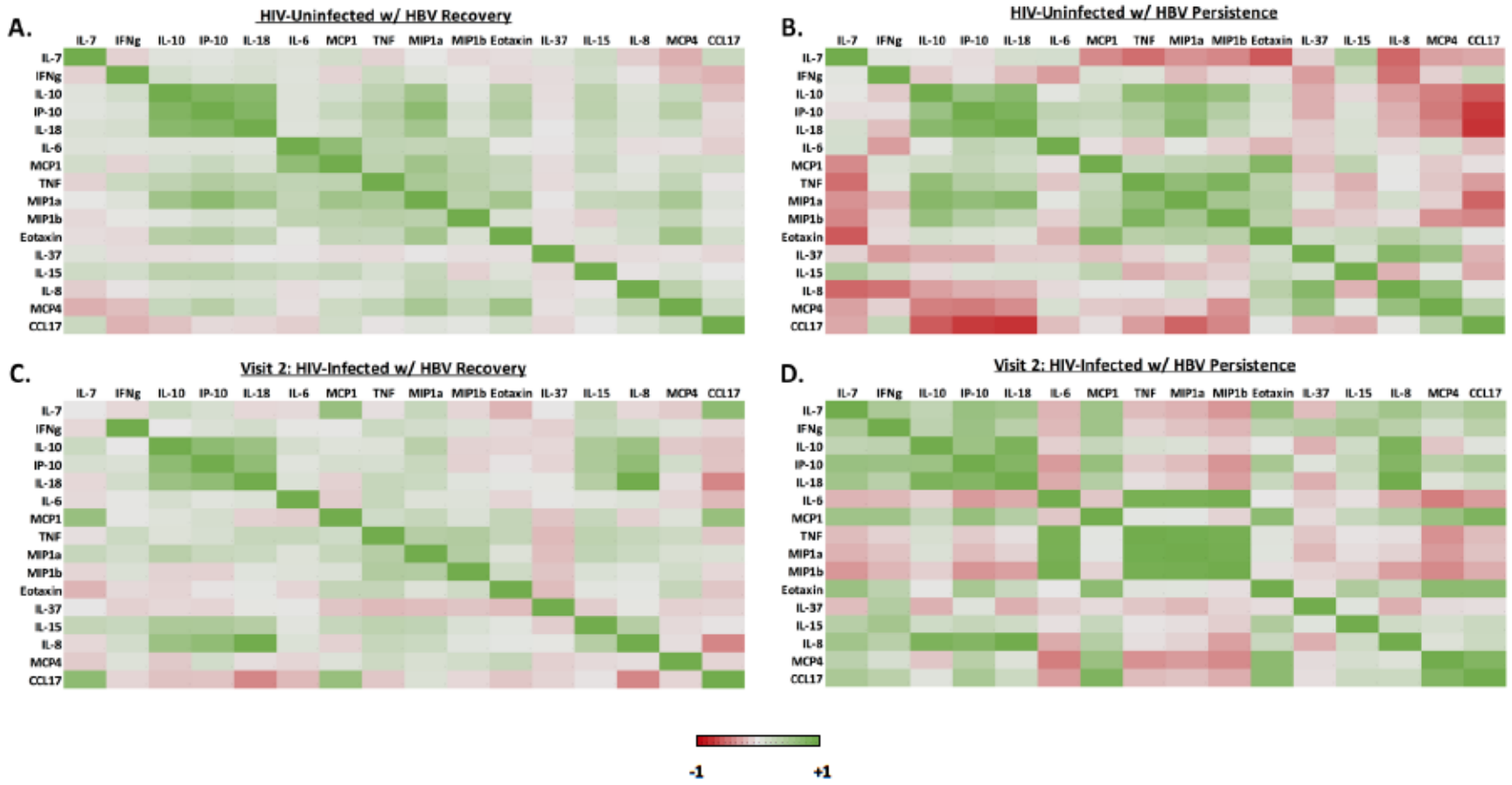


Table 4.10: Factor Loading Values from Principal Component Analysis

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
MIP1α	0.85	-0.21	-0.05	0.27	-0.17
TNF	0.82	-0.19	-0.12	0.13	-0.19
IP-10	0.75	0.43	0.12	-0.21	0.19
MIP1β	0.74	-0.36	-0.18	0.28	-0.21
IL-10	0.71	0.44	-0.13	-0.13	0.13
IL-18	0.65	0.52	-0.11	-0.18	0.23
Eotaxin	0.59	-0.39	0.22	-0.34	0.23
IL-8	0.53	-0.39	-0.29	0.21	-0.11
MCP4	0.44	-0.52	0.26	-0.27	0.37
MCP1	0.41	-0.05	0.71	0.06	-0.14
IFNγ	0.31	0.45	-0.08	-0.17	-0.32
IL-15	0.26	0.44	0.40	0.16	-0.15
IL-37	0.00	0.03	0.00	0.60	0.70
IL-7	-0.01	0.43	0.51	0.43	-0.05
CCL17	-0.17	-0.38	0.68	-0.09	-0.10
Variance Explained, %	30.9	14.5	11.1	7.3	7.0
Cumulative Variance, %	**30.9**	**45.4**	**56.5**	**63.8**	**70.8**

Figure 4.6: Loading Factors of Cytokines/ Chemokines for Immune Factors 1 and 2.

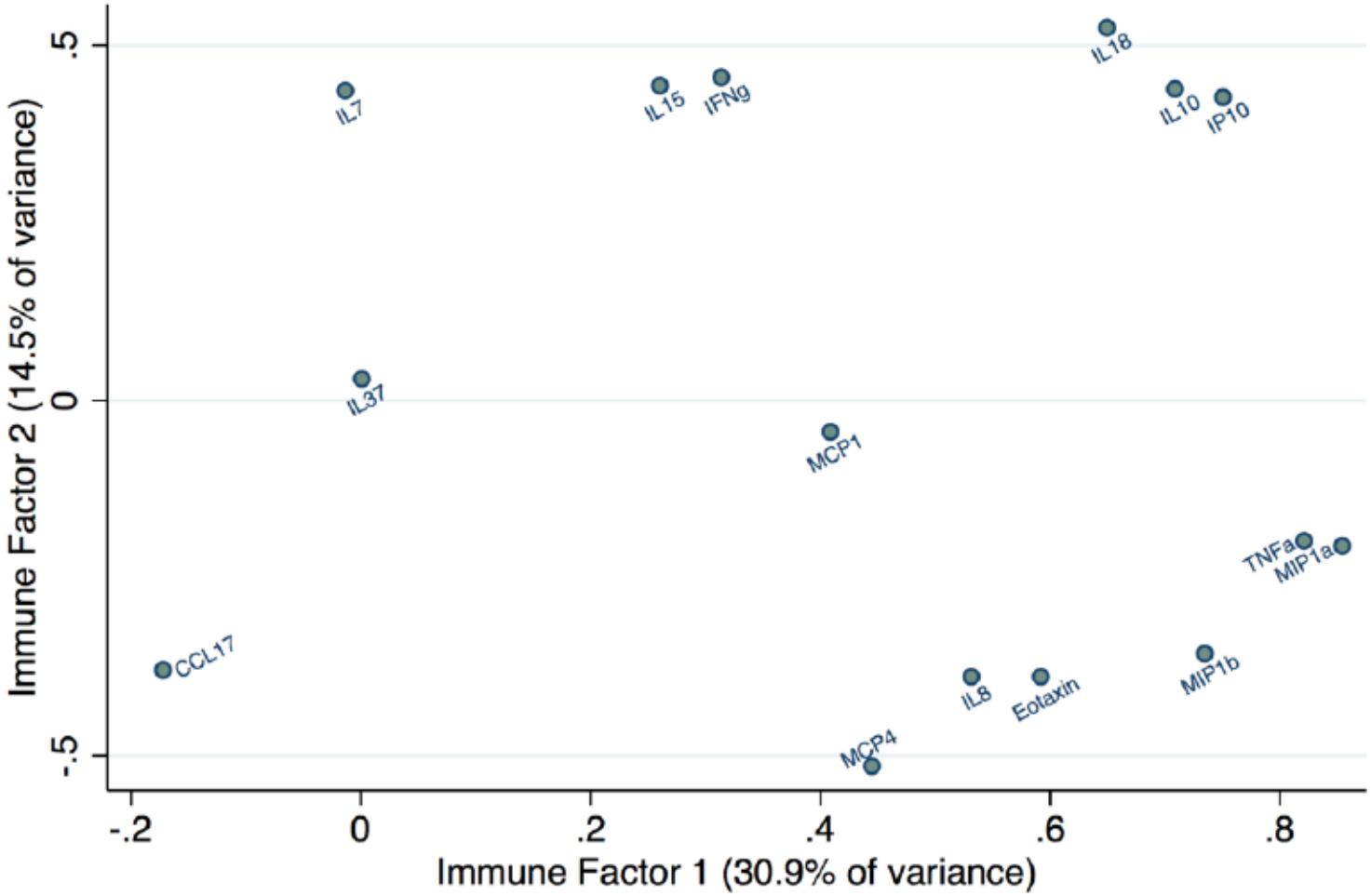


Figure 4.7: PCA Plot by HBV outcome and AST

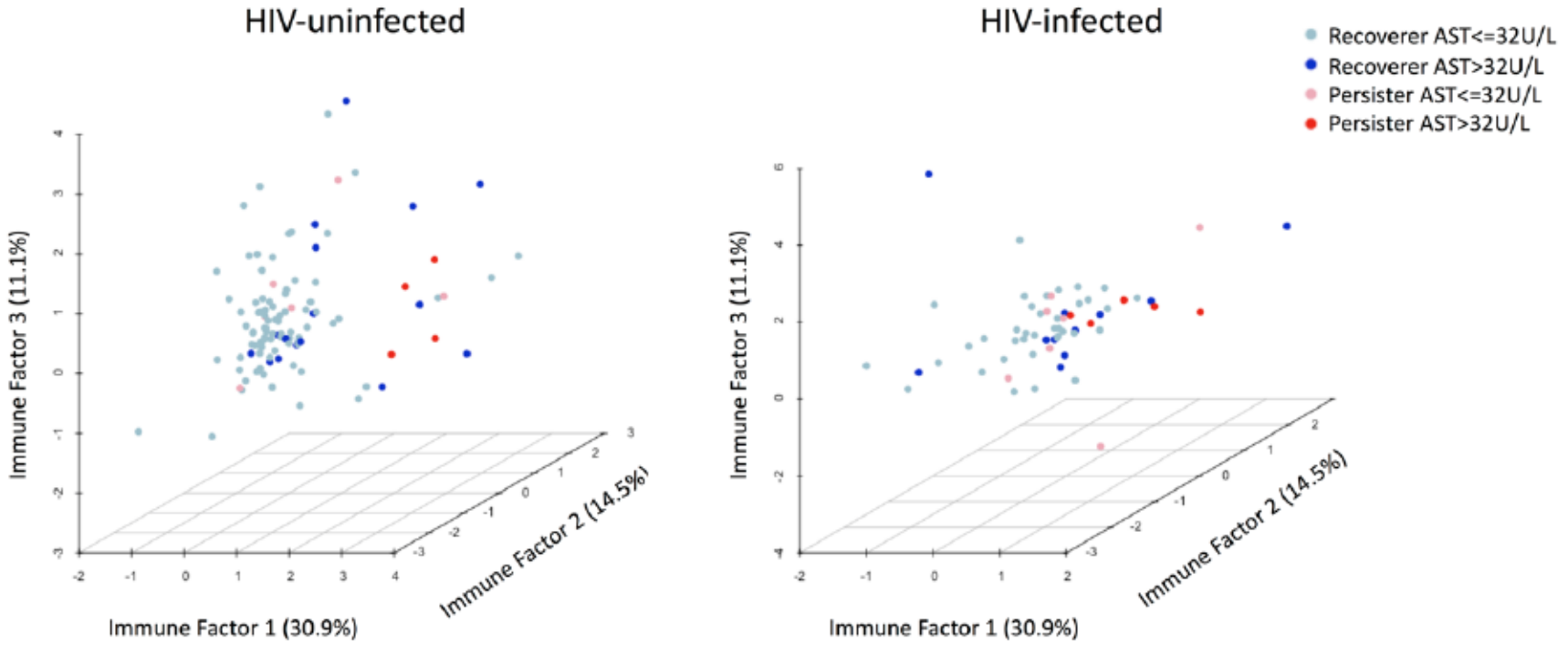


Table 4.11: Distribution of Individuals in Acute Infection Stages

	EA Stage (HBsAg+/ anti-HBc-)	IAA Stage (HBsAg+/ anti-HBc+)	SSC Stage (HBsAg-/ anti-HBc+)	Total
HIV-uninfected Recovery				
N	12	10	78	100
AST (U/L)	20.5 (13.5-39.3)	21.8 (5.0-243.8)	18.0 (14.0-27.1)	18.0 (13.5-29.1)
HBV DNA (log10 copies/ml)	3.8 (3.1-6.2), n=8	4.4 (3.2-6.2), n=8		4.07 (3.2-6.2), n=16
HIV-uninfected Persistence				
N	5	5	N/A	10
AST (U/L)	12.1 (11.0-21.8)	158.8 (131.0-260.1)		24.9 (12.1-158.8)
HBV DNA (log10 copies/ml)	7.2, n=1	7.9 (7.3-7.9), n=4		7.6 (7.2-7.9), n=6
HIV-infected Recovery				
N	4	7	40	51
AST (U/L)	16.1 (11.6-20.6)	22.5 (23.2-160.4)	21.7 (25.4-27.6)	22.5 (16.1-28.6)
HBV DNA (log10 copies/ml)	3.0 (2.0-4.0), n=2	6.6 (6.1=7.1), n=4		5.0 (3.0-6.6), n=4
HIV-infected Persistence				
N	5	3	N/A	12
AST (U/L)	28.3 (13.8-34.1)	23.1 (5.0-268.0)		25.7 (13.7-55.9)
HBV DNA (log10 copies/ml)	3.0 (2.4-5.5), n=3	8.3 (6.7-8.4), n=3		7.5 (4.3-8.4), n=8

Figure 4.8: PCA Plot by HBV outcome and acute infection stage

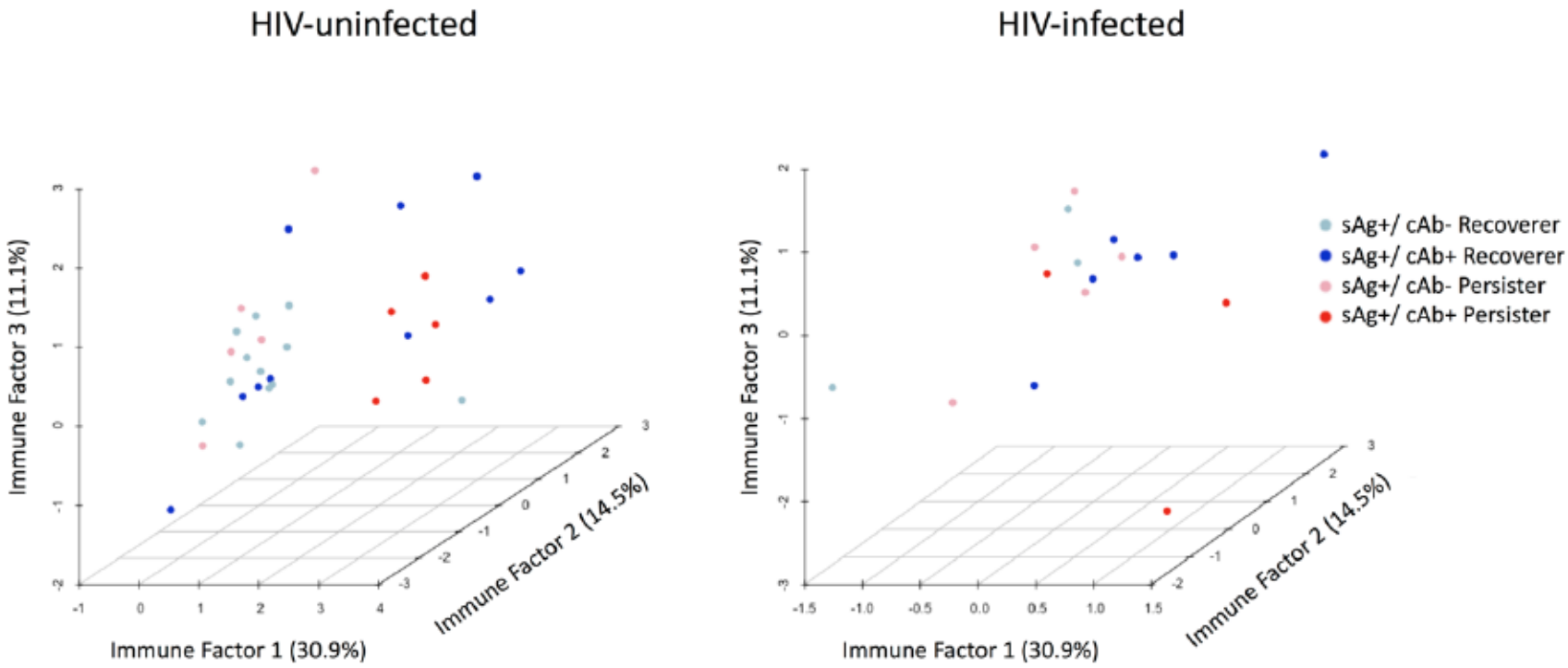


Table 4.12: Circulating Cytokine/ Chemokine Changes by Acute Infection Stage in HIV-uninfected Individuals with HBV Recovery

Values represent Median (Interquartile Range) of same-person visit 2 fold-changes from baseline. P Values from Kruskall-Wallis tests comparing individuals within the three stages of acute infection. Bold text indicates group is significantly different ($p<0.05$) than the SSC comparison group. * and bold text indicate group is significantly different ($p<0.05$) than both EA and SSC comparison groups.

	EA Stage HBsAg+/Anti-HBc- (n=12)	IAA Stage HBsAg+/Anti-HBc+ (n=10)	SSC Stage HBsAg-/Anti-HBc+ (n=77)	p value
IFNγ	1.0 (1.00-1.00)	1.0 (1.00-1.00)	1.0 (1.00-1.00)	0.481
IL-7	0.98 (0.72-1.10)	1.05 (0.98-1.18)	0.95 (0.78-1.22)	0.551
IL-8	0.97 (0.65-1.99)	1.65 (0.70-2.87)	0.90 (0.55-1.56)	0.239
IL-10	1.00 (1.00-1.14)	**4.37 (1.00-25.20)***	1.00 (1.00-2.00	**0.014**
IL-15	1.00 (0.95-1.04)	1.03 (0.98-1.31)	0.99 (0.92-1.10)	0.588
IL-18	1.11 (0.93-1.39)	**2.20 (1.25-3.76)**	1.06 (0.90-1.19)	**0.007**
IL-37	1.00 (0.93-1.00)	1.00 (0.94-1.20)	1.00 (0.96-1.02)	0.727
TNF	1.30 (0.89-2.31)	**1.73 (1.26-2.46)**	1.03 (0.89-1.38)	**0.003**
IP10	1.14 (0.84-1.92)	**5.23 (1.15-21.43)***	1.10 (0.84-1.34)	**0.009**
Eotaxin	0.97 (0.86-1.11)	1.19 (0.89-1.48)	1.04 (0.82-1.28)	0.406
MCP-1	1.10 (0.95-1.32)	1.17 (0.94-1.46)	1.02 (0.85-1.15)	0.114
MCP-4	1.01 (0.88-1.11)	1.12 (0.88-1.39)	0.97 (0.81-1.17)	0.328
MIP1α	**1.31 (0.96-1.49)**	**2.03 (1.12-3.04)**	0.99 (0.79-1.19)	**0.002**
MIP1β	**1.39 (1.12-2.74)**	**1.81 (1.50-4.70)**	0.96(0.68-1.51)	**0.022**
CCL17	0.88 (0.75-1.08)	1.01 (0.92-1.29)	0.97 (0.87-1.13)	0.512
AST	20.5 (13.5-39.3)	21.8 (5.0-243.8)	18.0 (14.0-27.1)	0.799

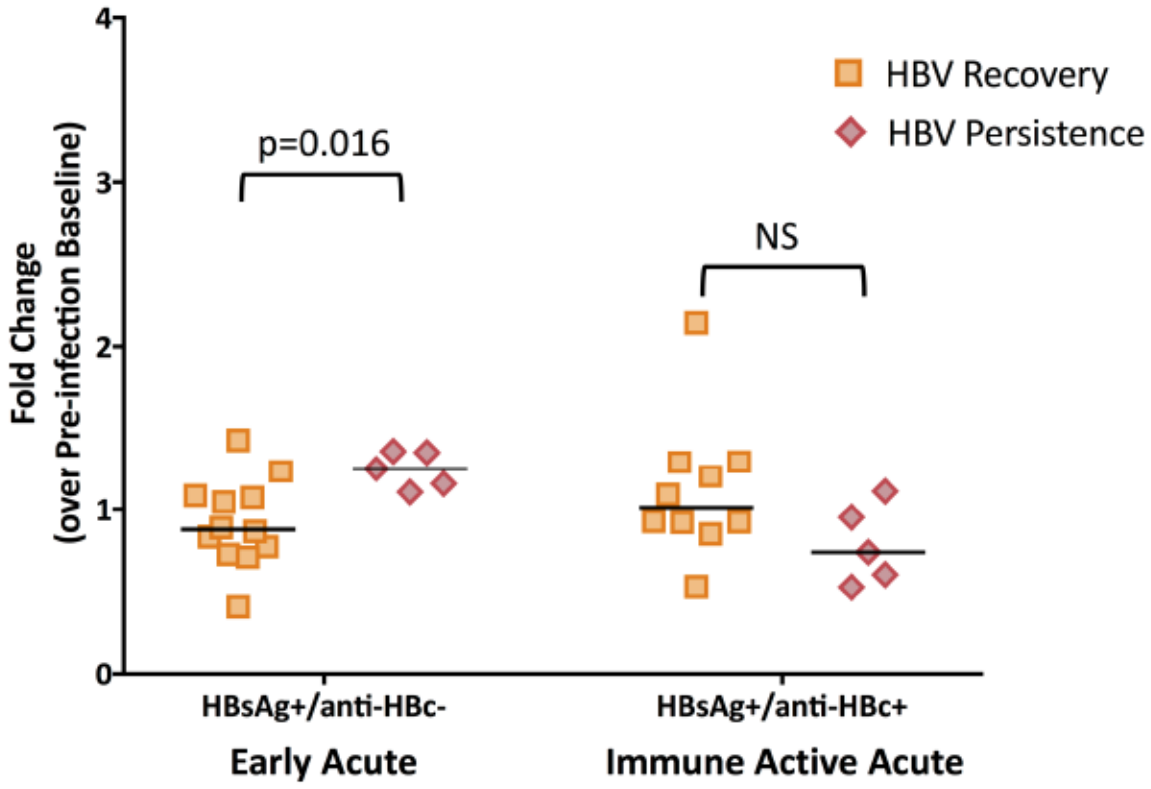
Table 4.13: Circulating Cytokine/ Chemokine Changes by Acute Infection Stage in HIV-uninfected Individuals

Values represent median (IQR) of same-person visit 2 fold-changes from baseline. P Values from univariate rank-sum tests, stratified by acute infection stage, comparing individuals who later experience HBV recovery versus those that develop persistent infection following incident HBV infection. Bold font indicates significant differences (P<0.05) identified by univariate analysis.

	Early Acute (EA) HBsAg+/Anti-HBc-			Immune Active Acute (IAA) HBsAg+/Anti-HBc+		
	HBV Recovery (n=12)	HBV Persistence (n=5)	p value	HBV Recovery (n=10)	HBV Persistence (n=5)	p value
IFNγ	1.0 (1.00-1.00)	1.00 (0.49-1.00)	0.324	1.00 (1.00-1.00)	1.00 (0.91-1.00)	0.189
IL-7	0.98 (0.72-1.10)	1.30 (1.22-1.41)	0.171	1.05 (0.98-1.18)	1.25 (1.01-1.45)	0.391
IL-8	0.97 (0.65-1.99)	1.33 (1.09-2.75)	0.343	1.65 (0.70-2.87)	1.60 (1.42-2.27)	1.000
IL-10	1.00 (1.00-1.14)	2.13 (1.00-2.53)	0.445	4.37 (1.00-25.20)	17.80 (12.56-24.80)	0.219
IL-15	1.00 (0.95-1.04)	0.94 (0.86-1.05)	0.527	1.03 (0.98-1.31)	1.00 (0.85-1.11)	0.540
IL-18	1.11 (0.93-1.39)	1.11 (1.01-1.26)	0.916	2.20 (1.25-3.76)	3.04 (2.82-3.51)	0.327
IL-37	1.00 (0.93-1.00)	1.00 (1.00-1.49)	0.382	1.00 (0.94-1.20)	1.13 (1.00-1.33)	0.461
TNF	1.30 (0.89-2.31)	1.24 (1.03-1.50)	0.752	1.73 (1.26-2.46)	1.82 (1.49-2.12)	1.000
IP10	1.14 (0.84-1.92)	1.06 (0.86-1.10)	0.461	5.23 (1.15-21.43)	7.96 (5.32-8.40)	0.903
Eotaxin	0.97 (0.86-1.11)	1.05 (0.75-1.82)	0.752	1.19 (0.89-1.48)	1.26 (0.75-1.33)	0.462
MCP-1	1.10 (0.95-1.32)	1.09 (1.04-1.28)	0.461	1.17 (0.94-1.46)	1.21 (0.99-1.46)	0.807
MCP-4	1.01 (0.88-1.11)	1.03 (0.87-1.34)	0.673	1.12 (0.88-1.39)	0.86 (0.65-0.95)	0.050
MIP1α	1.31 (0.96-1.49)	1.35 (0.86-1.35)	0.752	2.03 (1.12-3.04)	3.20 (2.45-3.34)	0.142
MIP1β	1.39 (1.12-2.74)	1.01 (0.97-1.51)	0.343	1.81 (1.50-4.70)	3.12 (1.57-4.43)	0.713
CCL17	**0.88 (0.75-1.08)**	**1.25 (1.16-1.36)**	***0.015***	1.01 (0.92-1.29)	0.74 (0.60-0.95)	0.142

Figure 4.9: Visit 2 CCL17 in HIV-uninfected individuals in Early Acute and Immune Active Acute Stages.

Each Symbol represents an individual. NS= Not Significant, determined by Rank-sum test.



CHAPTER 5:

Perspectives and Future Directions

5.1 A New Call for a Cure to Hepatitis B

Chronic hepatitis B is estimated to affect over 250 million people worldwide, and is a leading-cause of liver-related morbidity and mortality, responsible for an estimated 885,000 deaths annually [2]. HBV is a highly transmissible enveloped DNA virus that can persist outside of the body for over a week. While rates of transmission have historically been greatest in endemic areas where perinatal transmission is common, it has been shown here (Chapter 2) and elsewhere that administration of the birth-dose of the recombinant HBsAg vaccine is highly effective at lowering rates of CHB. Unfortunately, coverage rates of birth-dose vaccination are estimated to be only 39% worldwide, and even in areas with high coverage, incident infection still occurs in children[2,257].

In the past few years, a new challenge in HBV reduction has surfaced, as the rise of opioid use in the United States is accompanied by the first increase in acute HBV infection since the introduction of the vaccine over 20 years ago. In Pasco, Florida, rates of acute HBV-infection rose significantly from 1.5 to 17.28 per 100,000 people from 2011 to 2016 ($p<0.001$), and largely affected individuals 30-50 years who used injection drugs[5]. Because incident HBV infection often does not manifest in acute clinical symptoms, rates of incident HBV infection are likely higher in this population, and many individuals may be unknowingly transmitting HBV. Worldwide, only 9% of individuals with CHB infection are aware of their status[2]; within the Tibetan community assessed in chapter 2, only 58 (23.6%) of the 247 individuals with CHB were aware of their status. Better public health campaigns are needed to raise awareness of hepatitis B and

increase testing coverage, especially amongst populations with growing incidence and prevalence.

For those that become chronically infected, treatment with current antiviral regimens targeting the viral reverse transcriptase are life-long, as spontaneous recovery is rare and withdrawal from antivirals has been associated with viral rebound and severe, life-threatening hepatitis[291,292]. Given that these treatments are often unaffordable for those that need them in developing countries or amongst the uninsured, individuals may risk taking them intermittently or using less than the recommended dose to save money, which poses an additional risk for emergence of drug-resistant strains. Within the Tibetan community assessed in Chapter 2, 32 (55%) of the 58 individuals who were aware of their CHB had tried traditional Tibetan medicines, while only 10(17%) had reported previous use of antiretrovirals, citing cost as the limiting factor for not using antiretrovirals.

Finding a cure for hepatitis B infection would eliminate the need for life-long treatment with antiretrovirals, lower HBV-related morbidity and mortality, and reduce transmission of HBV infection. However, cure is challenging as the current canon on natural recovery is that the HBV cccDNA persists within hepatocytes of individuals following recovery because it is immune-mediated control rather than complete eradication of HBV from hepatocytes that leads to recovery[42]. This is supported by evidence showing reactivation of HBV replication following administration of immunosuppressive chemotherapy many years after recovery[24,25,283,285]. While new gene-editing technologies (e.g. CRISPR) have made it more plausible for a cure that fully

eradicates HBV by targeting HBV cccDNA for degradation, the *in vivo* effectiveness of these strategies and consequences of off-target effects are not well characterized[293–295]. Hence, a functional cure that mimics natural recovery, rather than a total cure that eliminates cccDNA from infected hepatocytes, may be a more realistic goal. As such, a more comprehensive understanding of the immune mechanisms underlying recovery is needed to effectively target the appropriate arms of the immune system. This thesis adds to the current understanding of immune responses in acute HBV infection by: 1) identifying a role for *Ccr5* deficiency in enhancing innate intrahepatic immune cell recruitment in a murine model of acute HBV, and 2) identifying a potential role for CCR4-mediated signaling in the acute phase of persistent infection, thereby offering a potential mechanistic explanation for development of persistent infection.

5.2 *Ccr5* deficiency enhances innate immune responses in the liver in a murine model of acute HBV

Human studies previously identified a link between the 32-bp deletion in CCR5 (CCR5Δ32), which results in a non-functional receptor, and enhanced recovery from acute HBV[236]. To investigate the role of *Ccr5* deficiency in immune cell trafficking into the liver in acute HBV infection, a murine model was employed (Chapter 3), in which it was found that *Ccr5* deficiency led to an enhanced innate immune cell recruitment into the liver during the early (day 3) and later stages (day 14) of acute infection. During early acute infection, a large population of infiltrating NK cells was present in *Ccr5*-deficient mice as compared to WT mice. Evidence was presented to suggest these NK

cells were recruited via the CXCR3/CXCL10 (IP-10) axis, which is hypothesized to be the result of enhanced sensitivity to CXCR3-mediated chemotaxis in the absence of CCR5 signaling.

Later in acute infection, greater serum ALT was associated with an infiltrating population of pro-inflammatory monocytes and increased IL1β in *Ccr5* deficient mice. In this experimental design, the mice were not studied past 14 days, so it is unknown whether *Ccr5* deficiency was associated with known immune correlates of improved recovery in humans, including development of protective antibody responses against HBsAg and HBV-specific T cell responses. However, murine and chimpanzee studies by other investigators have identified a role for NK cells in improving immune responses in HBV and other viral infections either directly via production of antiviral cytokines (TNF and IFNγ) or indirectly via modulation of the adaptive T cell response[102,146,296]. Further, murine studies by Publicover et al. (2013) and Huang et al. (2013) have identified a key role for pro-inflammatory monocytes in the formation of intrahepatic immune clusters, which prime productive T cell and antibody responses important for recovery from acute infection[160,282]. Taken together, these findings suggest *Ccr5*-deficient mice may exhibit better long-term control in this model of acute HBV infection, but a longer post-infection follow-up would be required to validate this hypothesis.

This study was initially undertaken with the hypothesis that *Ccr5* deficiency would impair regulatory T cell recruitment into the liver, and hence would improve HBV-specific adaptive immunity and recovery. Instead, it was found that *Ccr5* deficiency enhanced intrahepatic accumulation of innate cells that have been associated with

improved adaptive responses and recovery in other murine studies. In doing so, it provides a potential mechanistic explanation for the enhanced recovery from acute HBV observed in humans lacking CCR5 signaling, and suggests CCR5 blockade with the FDA-approved drug maraviroc may be beneficial in treatment of acute HBV infection.

5.3 Peripheral CCL17 is elevated in HBsAg+ individuals who develop CHB before development of antibodies against HBcAg

Most studies of HBV infection conducted in humans capture individuals with acute symptomatic presentation in clinics or after development of CHB; the number of individuals captured in early acute infection are low, and lack baseline cytokine values for comparison[120,150,153,180,182,188,191,206,228,297,298]. Hence, cytokine changes associated with the earliest stages of acute infection are not fully characterized. Further, it remains unclear whether the immune dysfunction associated with CHB occurs during the acute phase and leads to development of persistent infection, or if chronic antigen exposure resulting from persistent infection leads to the immune dysfunction observed in CHB. To address this gap in knowledge, an examination of peripheral cytokines and chemokines was undertaken in 173 men with incident HBV infection identified serologically within the larger MACS cohort (Chapter 4). In the 110 HIV-uninfected individuals, of whom ten developed chronic infection, it was found that the majority of the measured cytokine profiles were shared between individuals who recovered from HBV infection and individuals who developed chronic infection. In both cases, the cytokine profiles changed according to the stage of incident infection, defined

serologically by the presence of HBsAg and anti-HBc antibodies, rather than by final outcome of incident infection. In particular, the earliest stage of acute infection (HBsAg+/ anti-HBc-) was associated with increased MIP1α and MIP1β; HBsAg+ individuals with anti-HBc antibodies had further elevated levels of MIP1α and MIP1β, as well as increased TNF, IL-18, IP-10, and IL-10. The finding that individuals who develop chronic infection share much of the pro-inflammatory profile observed in individuals who recover from HBV infection was in contrast to what was hypothesized based on previous *in vitro* and *in vivo* studies in human and animal models, which suggested individuals who recovered from incident HBV infection would have greater pro-inflammatory and antiviral cytokine profiles (IL-12, TNF, IFNγ, IL18) than individuals who developed chronic infection. Interestingly, while most of the cytokine changes were not significantly different between individuals who recovered and those that developed chronic infection during early incident HBV, CCL17 expression patterns were altered in individuals who developed chronic infection, with elevated levels observed prior to the development of anti-HBc antibodies and decreased (below baseline) levels observed at follow-up 6-12 months later. Individuals who recovered did not follow this pattern.

CCL17 and CCL22 are chemokines that binds to the receptors CCR4 and CCR8 on Th2, Th17, regulatory T cells, and in the case of CCR8, myeloid cells that induce regulatory T cells[299–302]. Of note, the chemotaxis in response to CCL17 and CCL22 is important for recruitment of regulatory T cells to sites of immune clusters within the liver microenvironment [276]. The finding that CCL17 is elevated in early chronic infection suggests T cell recruitment may be shifted away from the classic Th1 phenotype which

has been associated with recovery[97,98], and provides a potential mechanistic explanation for development of persistent infection.

This study offers a novel contribution to the field by characterizing immune dynamics in early HBV infection in populations that are understudied, namely asymptomatic incident HBV infection and incident infection that leads to CHB. While the study design was comprehensive in its inclusion of 24 different cytokines/ chemokines, it is possible that other immune markers that were not measured (or were undetectable in the periphery in this study but are important in autocrine/paracrine signaling within the liver) play important roles in early immune responses to HBV infection. Still, these findings pose many interesting questions about the role of CCL17/CCL22 and CCR4/ CCR8 in development of persistent HBV infection that will be addressed in the next section.

5.5 Future Directions

While the studies presented in this thesis provide a significant contribution to our knowledge of the immunobiology of early HBV infection, they also generate additional questions and reveal gaps in knowledge that should be addressed in future research.

In a murine model of acute HBV, *Ccr5* deficiency enhanced early recruitment of NK cells to the liver, highlighting the importance of NK cells in the immune response to HBV. Evidence suggests that this early NK cell recruitment was driven by enhanced sensitivity of NK cells from *Ccr5*-deficient mice to IP-10/CXCR3-mediated chemotaxis during acute HBV infection, but it remains to be proven: Do NK cells from *Ccr5-/-* mice have enhanced chemotaxis in response to IP-10 compared to $Ccr5^{+/+}$ mice? Are other cells that express

CXCR3, such as T cells, also more sensitive to IP-10 mediated chemotaxis in *Ccr5* deficient mice? In order to address these questions, an *in vitro* transwell assay can be used to quickly and inexpensively measure immune cell chemotaxis of splenocytes from *Ccr5* WT and *Ccr5*-deficient mice in response to IP-10. As it is hypothesized that CCR5 signaling will diminish CXCR3 sensitivity, this experiment should be performed with and without pre-treatment of splenocytes with the three CCR5 ligands (CCL3, CCL4, CCL5). It is hypothesized that a greater number of NK cells from *Ccr5*$^{-/-}$ mice will migrate in response to IP-10 due to an increased sensitivity towards CXCR3 mediated chemotaxis in the absence of CCR5 signaling; further, it is hypothesized that NK cells from *Ccr5* WT mice will be less responsive to IP-10 chemotaxis following pre-incubation with CCR5 ligands.

These *in vitro* assays should be complemented by *in vivo* studies using antibody-mediated blockade of CXCR3 in *Ccr5*$^{-/-}$ mice, as well as chemotherapeutic (e.g. maraviroc) or antibody-mediated blockade of CCR5 with and without antibody blockade of CXCR3 in WT mice. It is hypothesized that CCR5 antagonism in *Ccr5* WT mice will lead to early NK cell recruitment into the liver in acute HBV, consistent with findings in *Ccr5*-deficient mice. Further, it is hypothesized that CXCR3 blockade in *Ccr5*$^{-/-}$ or in combination with CCR5 antagonism in *Ccr5* WT mice will decrease early NK cell recruitment into the liver in acute HBV.

Later in acute HBV, *Ccr5* deficiency leads to enhanced recruitment of CD11b^{+} Ly6cHi monocytes into the liver. It is hypothesized that this occurs along the CCL5/CCR1 axis, consistent with findings in a ConA model of autoimmune hepatitis[239]. Further, it is

hypothesized that these pro-inflammatory monocytes may be forming intrahepatic foci of innate and adaptive cells that lead to enhanced priming of T cells and antibody responses, consistent with formation of iMATEs[160]. In order to prove the first of these hypotheses, this model of acute HBV should be expanded into *Ccr1*$^{-/-}$ mice, and chemotherapeutic antagonism of Ccr5 performed in both WT and Ccr1$^{-/-}$ mice. Further, *in vivo* antibody blockade of CCR5 ligands or siRNA-knockdown of *Ccl3*, *Ccl4*, and *Ccl5* should be performed in *Ccr5*$^{-/-}$ mice infected with AdHBV. It is hypothesized that a CD11b^{+}Ly6cHi monocyte population would be observed in later acute HBV in WT mice following CCR5 antagonism, consistent with findings in *Ccr5*$^{-/-}$ mice, but would not be observed in the liver of *Ccr1*$^{-/-}$ mice, regardless of CCR5 antagonism. It is also hypothesized that CCL5 blockade, but not CCL3 or CCL4 blockade, will diminish the infiltration of this pro-inflammatory monocyte population observed in *Ccr5*-deficient mice.

To test the hypothesis that these CD11b^{+} Ly6cHi monocytes present within the liver at day 14 in *Ccr5*-deficient mice form immune foci key to functional intrahepatic priming of adaptive responses, immunohistochemistry should be utilized to assess co-localization of the infiltrating monocyte population with intrahepatic T cells. Functional testing of HBV-specific responses in intrahepatic T cells by either intracellular cytokine staining (ICS) for IFNγ and TNF, or ELISpot detection of IFNγ-producing cells, should be performed later in infection with and without *in vivo* clodronate depletion of monocytes/ macrophages at days 10-14 days post-AdHBV infection in *Ccr5*$^{-/-}$ mice. It is hypothesized that T cells will be localized to foci within the liver with CD11b+ monocytes

in *Ccr5* deficient mice, and HBV-specific T cell responses will be more robust by day 20 in these mice. Further, it is hypothesized that functional HBV-specific T cell responses would be decreased following clodronate depletion of intrahepatic monocytes/ macrophages. Finally, it would be prudent to assess whether CCR5 antagonism in a chronic AdHBV model would lead to recruitment of pro-inflammatory monocytes and enhance recovery in a chronic model.

From the study of incident HBV infection in humans, the finding that CCL17 was increased in early CHB raises further questions about the early immune response in HBV infection and the contribution of T cell skewing towards development of persistent HBV. Among these, what is the cellular source of CCL17? What happens if CCL17 or CCR4 signaling is blocked? What role does CCL22, the second ligand for CCR4, play in incident HBV infection? Can the findings from humans be modeled in an animal model for further study?

An important first step towards elucidating the role of CCR4-mediated chemotaxis in incident HBV infection would be to assay circulating CCL22 in this cohort, as CCL22 also binds CCR4 to recruit regulatory T cells[303,304]. It is hypothesized that CCL22 expression would also be increased in this cohort.

CCL17 is produced primarily by myeloid derived DCs (mDC) in the intrahepatic microenvironment; CCL22, a second ligand for CCR4, can be produced by myeloid cells and hepatocytes. CCL22 in hepatoma cell lines by TGFβ, a cytokine produced by Kupffer cells and present at elevated levels in the liver of those with CHB[303]. It is known that hepatitis B proteins direct innate responses, with HBcAg promoting IL-10 production in

myeloid cells, and HBsAg activating mDCs to produce IL-6 while inhibiting IFNα production in plasmacytoid DCs[224,305–307]. To address whether proteins from HBV, infectious HBV, or hepatocytes infected with HBV can trigger dendritic cells to express CCL17 or CCL22, DCs isolated from human PBMCs or murine livers should be treated with HBcAg, HBsAg, whole infectious HBV, or co-cultured with Hepg2-NTCP cells infected with HBV. Given *in vitro* studies showing CCL17 and CCL22 expression following co-culture of mDCs with a hepatoma line expressing HCV[278], it is hypothesized that HBV-infected HepG2-NTCP cells will induce CCL17 and CCL22 expression in mDCs, but it is unclear whether virus or HBV-proteins alone will be sufficient to activate expression. There is a documented association in murine and chimpanzee models between HBV inoculum size and development of chronic infection[92,185] (REF) Thus, it would be intriguing to consider whether the CCL17/ CCR4-axis mediates the relationship between inoculum size and development of chronicity. To address this, intrahepatic and peripheral CCL17 and CCL22 could be assayed by ELISA following AdHBV injection in a low-dose chronic, a medium dose acute, or a high-dose acute model of hepatitis B. Further, it would be important to phenotype intrahepatic T cells in these models in WT mice, which can be accomplished by flow cytometry with staining panels including antibodies against CCR4, CCR8, CD25, and Foxp3. Given the findings from WT mice at each dose, experiments should be expanded into *Ccr4*$^{-/-}$, *Ccr8*$^{-/-}$, or *Ccr4*$^{-/-}$/ *Ccr8*$^{-/-}$ mice, with particular emphasis on phenotyping of intrahepatic T cells and assessing outcomes of viral recovery. It is hypothesized that CCL17 and CCL22 production would be lowest in the medium-dose acute model in which productive adaptive responses precede

copious HBV-specific gene expression, and may be greatest at the earliest stages of infection in the high-dose model. Further, it is hypothesized that *Ccr4*-deficient mice would have lower regulatory T cell recruitment into the liver regardless of inoculating dose, and that *Ccr4*-deficiency may lead to recovery in the low-dose chronic model. If it is found that dose inoculum is associated with early production of CCL17 and CCL22, this provides additional evidence that inoculating dose may be responsible for development of persistent infection. Further it would suggest that greater emphasis be placed on testing and managing HBV DNA levels in individuals with CHB at risk for transmitting. Finally, it is important to consider CCR4 or CCR8 as a potential therapeutic target for treatment of CHB. While it has been reported that CHB is associated with increased CCL22[303], a link between CHB and increased intrahepatic $CCR4^+$ or $CCR8^+$ cells needs to be established. Using liver biopsies from individuals with CHB or individuals without liver disease (obtained during laparotomy for other reasons and confirmed to have normal liver histology), CCR4 and CCR8 expression could be assayed by immunohistochemistry, flow cytometry, or qPCR. Immunohistochemistry provides an advantage of staining for multiple markers (e.g. Foxp3, a marker of regulatory T cells, CCR4, and CCR8) while maintaining spatial context within the liver, while flow cytometry allows for phenotypic characterization of $CCR4^+$ or $CCR8^+$ cells on more parameters but lacks spatial context within the liver. Consistent with chronic HCV infection, it is hypothesized that CCR4 and CCR8 levels will be greater in livers from individuals with CHB, and their expression will be mostly restricted to intrahepatic $CD25^+Foxp3^+CD127^-$ regulatory T cells.

As a final expansion on the study of immune function in early HBV infection, the HBV-specific adaptive response, both T cell and antibody-mediated, requires further characterization in this cohort. HBV-specific T cells in individuals with CHB are characterized by an "exhausted" phenotype, including high levels of PD-1 expression and poor cytokine production upon stimulation[308,309]. It is unclear whether these individuals have impaired T cell responses early in chronic infection and contribute to development of persistent infection, or if these phenotypes develop only after persistent viral antigen exposure. In order to test this, a multi-parameter flow-cytometry panel has been developed, and will be used to perform functional intracellular cytokine staining assays in T cells following overlapping peptide stimulation spanning the full HBV genome. It is hypothesized that HBV-specific T cells, notably those targeting the HBV polymerase region and HBsAg, from individuals who develop chronic infection may have higher levels of PD-1 and impaired TNF and IFNγ production early following incident HBV infection. Further, it is hypothesized that greater numbers of IL-10 producing T cell and B cells will be observed in the early phase of HBV infection in those that develop chronic infection. A potential limitation of these studies will be that they are limited to peripheral immune cells; even if differences do exist between individuals who recover and those that develop persistent HBV infection, animal studies suggest that these differences are confined to intrahepatic immune cells at the earliest stages of infection, and thus may not be detectable peripherally[282]

5.5 Towards a cure

Recovery from HBV infection is characterized by the production of neutralizing antibodies against HBsAg and functional HBV-specific T cell responses, while CHB is associated with development of an impaired, or tolerogenic, innate and adaptive immune responses. More specifically, later stages of CHB are associated with an increased number of circulating regulatory T cells, higher PD-1 expression and impaired cytokine production in HBV-specific T cells, and an increased proportion of IL-10 producing B cells[120,279,308,310–312]. Further, NK cells from individuals with CHB have diminished capacity to produce cytokines (e.g. IFNγ) and display higher levels of TRAIL- and NKG2D- mediated killing and suppression of CD8- and CD4- T cells[134,313–315]. Finally, there is evidence that myeloid-derived cells from individuals with CHB are also impaired, as they have decreased expression of costimulatory molecules, increased production of IL-10, and decreased CpG-induced expression of IFNα in plasmacytoid dendritic cells (pDCs)[305,316].

While new therapies are under development to target the different stages of the viral lifecycle, the persistent nature of HBV cccDNA will require life-long administration of these new therapies in the absence of host immune control. To address this, several approaches have been taken to overcome the immune tolerance associated with CHB, many of which aim to enhance HBV-specific T cell functionality, including therapeutic vaccines (DNA and viral vector vaccines), checkpoint inhibitors, and *ex vivo* antigen-loading of dendritic cells[317,318]. However, while these approaches are successful in restoring peripheral HBV-specific T cell function, they are inconsistent in their ability to

lower HBV DNA and HBsAg, likely the result of a sustained immunotolerant environment in the liver.

Due to the limited effectiveness of T-cell targeted therapies in CHB, strategies that engage the innate arm to overcome adaptive tolerance are of particular interest. These approaches include strategies to improve antiviral responses in myeloid cells, including agonism of pattern recognition receptors (PRRs), and blocking NK-cell mediated repression of adaptive responses[134,319,320].

TLR agonism is a promising approach to control HBV infection, as *in vivo* and *in vitro* studies demonstrating anti-HBV effects of TLR 2, 3, 4, 7, 8, and 9 agonists[321,322]. TLR 2, 3 and 4 activation exerts direct antiviral effects on hepatocytes and leads to production of IL-6 and IP-10 and type-1 IFNs, while the effects of TLR7, 8 and 9 agonism are exerted via activation of non-parenchymal cells, including DCs and monocytes, and secretion of cytokines that either directly inhibit HBV replication/ gene expression or induce adaptive responses to control infection [321,323].

An oral agonist of TLR7, GS-9620 is currently under clinical trials for treatment of CHB. Administration of GS-9620 to infected woodchucks and chimpanzees overcame CHB-induced pDC suppression, led to endogenous IFNα production, reduced serum HBV DNA and HBsAg, and improved NK cell and T cell responses that persisted for many months following treatment[324,325]. Antibodies against HBsAg were produced within a portion of the woodchucks, and incidence of HCC was lowered from 71% to 8% compared to placebo-treatment[324]. Recent results on administration of GS-9620 in humans, however, did not alter serum HBsAg, but did improve NK cell and T cell function and

reduced NK-cell suppression of T cells, and it is suspected that the administered dose may be too low to achieve viral control[326].

While TLR9 agonism has not been explored in humans in the context of CHB, it is of particular interest that intravenous administration of the TLR9 agonist CpG in addition to therapeutic vaccination for HBcAg leads to formation of hepatic foci (termed iMATEs, or intracellular myeloid aggregates for T-cell expansion) in a murine model of CHB[160] . These immune cell aggregates were composed of $CD11b^+$ monocytes that facilitated robust HBcAg-specific CD8+T cell priming and expansion, and eliminated HBcAg production at day 42 following treatment in this chronic model[160].

The finding that $CD11b^+$ monocytes form foci that overcome T cell tolerance and facilitate HBV recovery in chronic infection is interesting given the observation that Ccr5 deficiency led to recruitment of pro-inflammatory $CD11b^+$ monocytes into the liver in the acute HBV model presented in chapter 3. The chemokine CCL5 binds to both CCR1, expressed at high levels on pro-inflammatory monocytes, and CCR5, which is not expressed on pro-inflammatory monocytes, but rather is expressed at high levels on anti-inflammatory monocytes[327]. Indeed, Moreno at al (2005) demonstrated in a ConA-model of autoimmune hepatitis that Ccr5 deficiency drove pro-inflammatory monocyte recruitment into the liver due to enhanced expression of Ccl5[239].

Ccl5 expression is high in liver biopsies from individuals with CHB[328,329], suggesting blockade of Ccr5-mediated trafficking would promote $CD11b^+$ monocyte accumulation in the liver in a chronic model of HBV infection, which may lead to improved intrahepatic T cell conditioning essential for mounting an appropriate HBV-specific T cell

and antibody-mediated response[282]. Blockade of Ccr5 with the FDA-approved maraviroc has recently been reported to improve outcomes and overcome immunosuppression in metastatic tumors within the liver, which are also associated with enhanced expression of CCL5[328]. Further, long-term maraviroc treatment in individuals with HIV/ HCV co-infection reduced hepatic fibrosis, suggesting a skew away from M2-polarized macrophages within the liver following treatment[330]. Taken together, these findings suggest consideration of maraviroc as a component of treatment for CHB deserves further attention

The finding that development of persistent HBV infection is associated with early increases in CCL17 suggests that greater recruitment of non-Th1 cells, and particularly regulatory T cells, into the liver may lead to persistent infection in humans. This is consistent with murine studies demonstrating that regulatory T cells suppress HBV-specific $CD8^+$ T cells within the liver microenvironment in acute HBV and lead to impaired recovery[139]. Therefore, antibody-mediated depletion of $CCR4^+$ and $CCR8^+$ regulatory T cells may provide a promising therapeutic approach to treatment of CHB by reducing the tolerogenic microenvironment of the liver, but requires further study. Regulatory T cell depletion with antibodies against CCR4 or CCR8 has considered as a potential chemotherapy in cancer, another condition characterized by immunosuppressive microenvironments, and depletion was associated with tumor inhibition in a murine model of breast cancer.[331] Still use of this approach should proceed with caution, as monoclonal therapy against CCR4 in treatment of T cell

lymphoma has been associated with reactivation of HBV long after recovery from HBV[283–285].

T cell based therapies that induce activation, rather than limiting inhibitory signals (e.g. PD1 blockade), capitalize on T cell plasticity and provide another potential therapeutic option for treatment of CHB. OX40 is a cell-surface receptor on the surface of T cells that receives activation signals via binding of OX40L on antigen-presenting cells[332]. The OX40L is significantly upregulated in iMATE clusters within the liver[160], and Publicover et al (2018) recently reported that OX40 agonism enhanced HBsAg seroclearance in a mouse model of CHB, suggesting activation of T cells via OX40 may assist in overcoming the immunotolerant liver microenvironment[333]. Further, studies have demonstrated that OX40 agonism suppresses regulatory T cells and enhances Th1 responses[333,334], making it another reasonable approach that deserves further exploration as part of combined therapy for CHB.

Because of the complex immune environment of chronic hepatitis infection, no single treatment is likely to result in recovery from CHB. Rather, a combination of therapies designed to overcome the immunotolerant nature of the liver microenvironment and induce appropriate immune cell priming in the liver will be important to developing an effective strategy for immune recovery in CHB. Further, it may be important to combine immune-based strategies with drugs that block or silence HBV transcription to reduce the immunomodulatory effects of HBV antigens, as antiviral treatment as been associated with partial restoration of immune responses. In total, the best strategy for a functional cure to chronic hepatitis B is likely one that combines therapeutic vaccination

(e.g. DNA vaccination w/ vector boost), antivirals, and immunotherapeutic agents like those discussed above that reprogram the tolerogenic innate and adaptive responses, to induce long-term T cell and antibody-mediated control of HBV.

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Appendix A: List of Abbreviations

HBV: Hepatitis B virus

HCC: Hepatocellular carcinoma

CHB: Chronic hepatitis B

HBsAg: Hepatitis B surface antigen

HBeAg: Hepatitis B envelope antigen

Anti-HBs: Antibodies against HBsAg

ALT: Alanine aminotransferase

AST: Aspartate Aminotransferase

cccDNA: Covalently-closed circular DNA

TLR: Toll-like receptor

HBIG: Hepatitis B immune globulin

IFN: Interferon

RT: Reverse transcriptase

rcDNA: relaxed circular DNA

NTCP: Sodium taurocholate cotransporting polypeptide

HBcAg: Hepatitis B core antigen

pgRNA: Pre-genomic RNA

HBV Pol: HBV polymerase

HBx: Hepatitis B x protein

SVP: Sub-viral particles

LSEC: liver sinusoidal epithelial cell

HSC: Hepatic stellate cells

IL: Interleukin

TNF: Tumor necrosis factor

LPS: lipopolysaccharide

APP: Acute phase proteins

CRP: C-reactive protein

DHBV: Duck hepatitis B virus

GSHV: Ground squirrel hepatitis virus

WHV: Woodchuck hepatitis virus

PHH: Primary human hepatocytes

MPCC: Micro-pattern co-culture

Anti-HBc: Antibodies against HBcAg

Anti-HBe: Antibodies against HBeAg

NK: Natural killer

ConA: concanavilin A

KO: Knock-out

WT: Wild-type

AASLD: American Association for the Study of Liver Diseases

IHL: Intrahepatic leukocytes

KRB: Krebs Ringer buffer

PBS: Phosphate buffered saline

BSA: Bovine-serum albumin

PMA: Phorbol 12-myristate 13-acetate

ICS: Intracellular cytokine staining

ELISA: Enzyme-linked immunosorbant assay

poly(I:C), polyriboinosinic-polyribocytidylic acid

GM-CSF: Granulocyte-macrophage colony-stimulating factor

DC: Dendritic cells

mDC: Myeloid dendritic cells

pDC: Plasmacytoid dendritic cells

PBMC: Peripheral blood mononuclear cells

LLOQ: Lower limit of quantification

PCA: Principal Component Analysis

PRR: Pattern recognition receptors

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EDUCATION

PhD, Johns Hopkins School of Public Health, Baltimore, MD, Molecular Microbiology and Immunology
Advisor: Chloe Thio, Johns Hopkins School of Medicine
Dissertation: Hepatitis B Infection: Immune Mechanisms Underlying Acute Infection

MS, Temple University, Philadelphia, PA, Epidemiology
Advisor: Steven Douglas, Children's Hospital of Philadelphia
Dissertation: Substance P and Neurokinin-1 expression in three brain regions of HIV-infected Individuals from the National NeuroAIDS Tissue Consortium Cohort: Findings & Implications of Drug Use and Viral Tropism in the Management of NeuroAIDS

BS, Carnegie Mellon University Pittsburgh, PA, Biological Sciences, minor in Political Science

RESEARCH EXPERIENCE

Johns Hopkins Bloomberg School of Public Health/ JHMI Baltimore, MD
Advisors: Chloe Thio and Bill Osburn November 2011-August 2018

- Building on the finding that CCR5 deficiency led to enhanced recovery in acute hepatitis B, utilized a murine model of acute HBV to identify a role for Ccr5 deficiency in enhancing intrahepatic innate cells associated with recovery.
- Utilizing a dataset and biological samples from the Multi-center AIDS Cohort Study(MACS), characterized the early immune dynamics associated with inflammation and development of chronic disease in a population of 110 individuals with incident HBV infection, of whom 9% developed chronic infection.

Johns Hopkins Bloomberg School of Public Health Baltimore, MD and Bylakuppe, India
Advisor: Christopher Hoffmann June 2013-January 2014

- Field Coordinator for a study of hepatitis B prevalence amongst three distinct populations within the Tibetan-in-exile population of Bylakuppe, India. Found 9% prevalence rate of CHB.
- Facilitated in development of study methods, including building of study survey, training manuals and field maps for random household sampling within accordance of Johns Hopkins IRB
- Performed data analysis and prepared scientific reports using STATA
- Trained and managed a team of six on-site Tibetan field nurses and hospital staff to conduct on-site questionnaires, serology testing, and hepatitis B education.

Children's Hospital of Philadelphia, Department of Infectious Disease Philadelphia, PA
Advisor: Michael Sebert May 2008-August 2011

- Identified a relationship between antibiotic-induced ribosomal errors and regulation of bacterial competence/ horizontal gene transfer in *Streptococcus pneumoniae*.

TEACHING EXPERIENCE

Johns Hopkins Bloomberg School of Public Health August 2012-August 2013, January- March 2016

- Teaching Assistant for: Principles of Immunology I and II, Immunology and Infection, Public Health Biology, and Immunology and Nutrition.

PRESENTATIONS

Stevens KE, Thio CL, Osburn WO. **Loss of CCR5 signaling results in early NK cell and neutrophil recruitment into the liver and increased hepatic inflammation in a mouse model of acute hepatitis B.** American Association of Immunologists Annual Meeting, Washington, DC, May 2017

Stevens KE, Zwack E**, Sebert ME. Modulation of pneumococcal competence in response to the accuracy of protein translation.** Cold Spring Harbor Labs, Molecular Genetics of Bacteria and Phages Cold Spring Harbor, NY, August 2010

POSTERS

Stevens KE, Thio CL, Osburn WO. **Loss of CCR5 signaling results in early NK cell recruitment into the liver and altered HBV-specific CD8+ T Cell function in a mouse model of acute hepatitis B.** HBV International Meeting, Washington, DC, September 2017

Stevens KE, Thio CL, Osburn WO. **Loss of CCR5 signaling results in early NK cell and neutrophil recruitment into the liver and increased hepatic inflammation in a mouse model of acute hepatitis B.** American Association of Immunologists Annual Meeting, Washington, DC, May 2017

Stevens KE, Thio CL, Osburn WO. **Loss of Ccr5 Signaling Results in Increased Liver Inflammation in a Mouse Model of Acute Hepatitis B.** HBV Treatment Endpoints Workshop: *From Discovery to Regulatory Approval*, Alexandria, VA, September 2016

Stevens KE, Hwang J, Thio CL, Osburn WO. **Influence of Ccr5 genotype on adaptive and innate gene expression in a mouse model of acute hepatitis B infection.** HBV International Meeting, Bad Nauheim, Germany, October 2015.

Stevens KE, Kowalko JE, Hammett A, Lee JY, Seeholzer SH, Sebert ME. **The HtrA protease digests the pneumococcal competence-stimulating peptide and contributes to the repression of competence.** American Society for Microbiology, General Meeting, Philadelphia, PA, May 2009.

PUBLICATIONS

Stevens KE, Thio CL, Osburn WO. (2018) **Ccr5 deficiency enhances hepatic innate immune cell recruitment and inflammation in a murine model of acute hepatitis B infection.** Submitted

Stevens K, Palmo T, Wangchuk T, et al. (2016) **Hepatitis B prevalence and treatment needs among Tibetan refugees residing in India**, *Journal of Medical Virology*, 88(8):1357-63.

Spitsin S, Stevens KE, Douglas SD. (2013) **Expression of substance P, neurokinin-1 receptor and immune markers in the brains of individuals with HIV- associated neuropathology**, *Journal of Neurological Science*, 334(1-2):18-23.

Gagne AL, Stevens KE, Cassone M, et al. (2013) **Competence in Streptococcus pneumoniae is a response to an increasing mutational burden**, *PLoS ONE*, 8(8):e72613.

Stevens KE and Sebert ME (2011) **Frequent beneficial mutations during single-colony serial transfer of Streptococcus pneumoniae**, *PLos Genetics* 7(8) :e1002232.

Stevens KE, Chang D, Zwack E, Sebert ME (2011) **Competence in Streptococcus pneumoniae is regulated by the rate of ribosomal decoding errors**. *MBio*, 2(5): e00071-11

SKILLS

- Molecular biology and cloning, gel electrophoresis, western blots, immunohistochemistry, ELISA, ELISpot, Flow Cytometry, functional T cell and NK cell assays, *in vivo* murine work, *in vitro* human and murine assays
- MS office, Prism, FlowJo, STATA, SPSS, R